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Antal Karl Hajos

Louisiana State University and Agricultural & Mechanical College

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**Metabolism and mutagenicity of dinitropyrenes: Effect of
chronic ethanol ingestion and Aroclor 1254 pretreatment**

Hajos, Antal Karl, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1991

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

**METABOLISM AND MUTAGENICITY OF DINITROPYRENES:
EFFECT OF CHRONIC ETHANOL INGESTION AND AROCLOR 1254
PRETREATMENT**

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biochemistry

by
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Dedication

To Monika

Foreword

This dissertation is divided into seven chapters. The first chapter provides an introduction to the origin of dinitropyrenes in our environment, followed by a summary of their metabolism in bacterial and mammalian cells as well as their mutagenic and carcinogenic activity. Subsequently, the effect of ethanol consumption as a modifier of xenobiotic metabolism, in particular that of nitroarenes, is discussed. Chapter two summarizes the effects of ethanol consumption on various drug-metabolizing enzyme systems, which may be potentially involved in the metabolism of dinitropyrenes. Chapter three is a manuscript entitled "Dinitropyrene nitroreductase activity of purified NAD(P)H-quinone oxidoreductase: role in rat liver cytosol and induction by Aroclor 1254 pretreatment", which identifies cytosolic NAD(P)H-quinone oxidoreductase as an inducible DNP-nitroreductase. This paper has been published in *Carcinogenesis*, volume 12 (4), p. 697-702, and has also been presented as a poster at the 74th meeting of the ASBMB in New Orleans, 1990. Chapter four continues on this subject in the form of a manuscript entitled "Purified NAD(P)H-quinone oxidoreductase potentiates the mutagenicity of 1,3-, 1,6- and 1,8-dinitropyrene." which investigates the role of NAD(P)H-quinone oxidoreductase in the mutagenic expression of dinitropyrenes. This work has also been presented as a poster at the 75th meeting of the ASBMB in Atlanta, 1991, and the contents of chapter three and four will be presented at the 13th meeting on polycyclic aromatic hydrocarbons in Bordeaux, France by Dr. Winston. Chapter five, which elucidates the role of this enzyme as well as that of alcohol dehydrogenase in the metabolism of C-nitroso compounds as a function of ethanol ingestion exemplifies the pertinent actions of ethanol as a modifier of nitro- and nitrosoarene metabolism. Chapter six reports on further considerations on the effects of ethanol ingestion in the metabolism of the nitroarenes under study, nitroreduction by the cytosolic fractions as

well as metabolism and genotoxic activity of dinitropyrenes are considered. Parts of this work have been presented as a poster at the International Winter Meeting 1989 of the Society for Free Radical Research in Tübingen, FRG. Finally, chapter seven is a summary of a theoretical approach employing the semiempirical molecular orbital method AM1 to investigate the chemical characteristics of nitrated pyrenes as well as that of their partially reduced metabolites. A short report identifying dicoumarol as an inhibitor of xanthine oxidase is contained in the appendix.

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List of Abbreviations

1-NAR	1-nitropyrene anion radical
1-NSP	1-nitrosopyrene
2-AA	2-aminoanthracene
2-AAF	2-acetylaminofluorene
2-AF	2-aminofluorene
4-NQO	4-nitroquinoline-N-oxide
1,3-NAR	1,3-dinitropyrene anion radical
1,3-NSNP	1-nitroso-3-nitropyrene
1,6-NAR	1,6-dinitropyrene anion radical
1,6-NSNP	1-nitroso-6-nitropyrene
1,8-NAR	1,8-dinitropyrene anion radical
1,8-NSNP	1-nitroso-8-nitropyrene
AcCoA	Acetyl Coenzyme A
AM1	Austin method 1
CBA	cibacron blue agarose
DC	dicoumarol
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNP	dinitropyrene(s)
DTPA	diethylenetriaminepentaacetic acid
EDTA	ethylenediaminetetraacetic acid
GSH	glutathione, reduced form
GST	glutathione-S-transferase
HPLC	high performance liquid chromatography
MFO	mixed function oxidase
MNDO	modified neglect of differential overlap
MOPAC	molecular orbital programs for analytical chemistry
NAD ⁺	β-nicotinamide adenine dinucleotide
NADH	β-nicotinamide adenine dinucleotide, reduced form
NADP ⁺	β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form

NATase	acetylCoA:arylamine N-acetyltransferase
NQOR	NAD(P)H:quinone oxidoreductase (DT-diaphorase)
PAH	polycyclic aromatic hydrocarbons
p-AP	p-aminophenol
p-NSP	p-nitrosophenol
pNP	p-nitrophenol
S9	9000 x g supernatant
SCF	self-consistent field
sCyt c	partially succinoylated ferricytochrome c
SD	standard deviation
SEM	standard error of the mean
STE	sucrose-Tris-EDTA
Tris	tris(hydroxymethyl)aminomethane
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
XD	xanthine dehydrogenase
XO	xanthine oxidase

Abstract

The nitroreduction of three potentially mutagenic dinitropyrene (DNP) isomers by liver cytosol from Aroclor-1254-pretreated rats was studied. NAD(P)H-quinone oxidoreductase (NQOR) is increased 12- to 24-fold by this treatment and resulted in up to 5-fold induction of cytosolic DNP-nitroreductase as compared to chow-fed controls. Upon purification, NQOR activity towards menadione coeluted with C-nitroso- and 1,6-DNP nitroreductase activity. Purified NQOR catalyzed NAD(P)H-dependent reduction of all DNP, displayed the same isomer specificity as did cytosol and increased the mutagenicity of all DNP in the Ames assay up to 3-fold. In TA98NR, a strain deficient in nitroreductase, only the mutagenicity of 1,3-DNP was markedly lowered. 1,6- and 1,8-DNP were activated to approximately equal extents by NQOR in TA98NR and TA98. NQOR potently increased the mutagenicity of 1,3-DNP in TA98NR to levels comparable to that obtained in TA98. NQOR increased the mutagenicity of 1,6- but not 1,3- and 1,8-DNP to mutagenic intermediates in TA98/1,8-DNP₆, a strain deficient in O-acetyltransferase activity. Ethanol ingestion caused a 3- to 5-fold increase in NQOR, but only a slight increase in DNP-nitroreductase activity. NQOR induction however, caused ~ 2-fold increases in the NAD(P)H-dependent rate of reduction of p-nitrosophenol. Inhibitor studies indicate a cofactor-specific participation of NQOR and alcohol dehydrogenase in the cytosolic C-nitrosoreductase activity. The genotoxicity of DNP towards *S. typhimurium* TA1535/pSK1002 was studied in the presence of cytosolic and microsomal fractions from ethanol- and pair-fed rats. Microsomes attenuated the genotoxicity of 1,3-, 1,6- and 1,8-DNP in the *umu*-test, the order being 1,3- > 1,6- > 1,8-DNP. Cytosol was deactivating 1,8-DNP most strongly, less with 1,6-DNP and activated 1,3-DNP. AM1 calculations for DNP and their reduced

derivatives indicate that the isomer selectivity displayed in the bioactivation of DNP is not solely based on chemical grounds.

Chapter 1

Introduction

1.1. Objective

The major objectives of this study were firstly to identify and characterize enzymatic activities involved in the bioactivation and mutagenic expression of dinitropyrenes (DNP) in rat liver subcellular fractions and secondly to evaluate the effect of ethanol consumption on the mutagenic expression of DNP and to identify and characterize those enzymes involved in DNP metabolism whose activity may be altered upon ethanol ingestion. Emphasis was also placed on the effect of pre-exposure of experimental animals to inducers of the microsomal mixed function oxidase (MFO) system since metabolism of many xenobiotics proceeds via cytochrome-P450-mediated reactions. Pretreatment of animals with MFO inducers commonly affects various other enzymes in the microsomal as well as in the cytosolic fraction. The contributions of both subcellular fractions to the metabolism and the mutagenicity of DNP were therefore considered.

Numerous studies have established that chronic alcohol consumption alters the metabolism, toxicity and mutagenicity of a wide variety of xenobiotic substances in

animal models. Further, there is strong epidemiological evidence for a link between alcoholism and human cancer risk. The present study is concerned with DNP, a class of ubiquitous environmental pollutants, which are considered to be some of the most potent bacterial mutagens and which are highly genotoxic in various animal models. While their existence in the environment and their extraordinary mutagenic potency in bacteria was documented almost a decade ago, relatively little is known of their metabolism in mammalian cells and subcellular fractions. The polychlorinated biphenyl (PCB) mixture Aroclor-1254 was employed in this study as animal pretreatment because Aroclor-1254 is a mixed inducer of the cytochrome P450 system and therefore is commonly employed when accessing the bacterial mutagenicity of xenobiotics and their possible activation by mammalian cell fractions.

1.2. Environmental occurrence of nitroarenes

Nitroarenes are ubiquitous contaminants of the environment. They are formed as a consequence of incomplete combustion processes and have arisen almost exclusively as a result of anthropogenic activity. Nitroarenes have been detected in extracts of diesel and gasoline emissions, fly ash particles, waste water from gasoline stations, used crankcase oil, cigarette smoke condensates, grilled foods and carbon black toners and have further been identified in emissions from incinerators, residential home heaters and wood burning stoves (Rosenkranz *et al.*, 1980; Rosenkranz, 1982; Nakagawa *et al.*, 1983; Rosenkranz and Mermelstein, 1983; 1985; Tokiwa *et al.*, 1987). Despite these numerous sources, studies have mainly concentrated on those nitroarenes from carbon black toners and diesel particulate matter. With regard to the former, the entire production process has had to be redesigned such that the formation of these

highly mutagenic products is greatly reduced (Rosenkranz *et al.*, 1980). In a comprehensive report on the sampling of vehicle emissions for chemical analysis and biological testing, Schuetzle (1983) reported on the identification of a moderately polar phase of diesel particulate matter which accounted for the majority of the total mutagenicity of diesel exhaust. This was indicated by the fact that 50-75% of the bacterial direct-acting mutagenicity¹ in *Salmonella typhimurium* TA98 of unfractionated diesel exhaust was found in this subfraction. Within this polar fraction, nitroarenes accounted for at least 40% of the total extract direct-acting mutagenicity. Mononitropyrenes, DNP and mononitrohydroxypyrenes were the most potent mutagens in this group. In summary, DNP are abundant in the environment and probably are of considerable risk to human health (Hecht and El-Bayoumy, 1989; IARC, 1990)

1.3. Mutagenicity, genotoxicity and carcinogenicity of dinitropyrenes

1.3.1. Bacterial mutagenicity of DNP in the Ames Salmonella mutagenicity and umu genotoxicity assay

Initial studies by Rosenkranz *et al.* (1983) have concluded that DNP do not react directly (i.e., without any further chemical activation) with DNA to yield covalent adducts. The Ames *Salmonella typhimurium* mutagenicity assay, which is based on reverse mutations of these bacteria, has been widely employed to study the mutagenicity

¹ The bioactivation of DNP requires only those enzymes already present in the bacteria. For this reason DNP are sometimes termed bacterial direct-acting mutagens. To distinguish the bacterial mutagenic response obtained in the absence of mammalian enzyme fractions from that obtained in their presence, the term direct-acting mutagenicity will be used throughout the remainder of this work. A more detailed description of the Ames *Salmonella* mutagenicity assay is given in section 1.3.1.

of DNP. The bacteria employed in the Ames *Salmonella typhimurium* mutagenicity assay are of the *his*⁻ genotype, that is, the gene which codes for the first enzyme of the histidine biosynthetic pathway had a defined mutation introduced at a specific site within it (Ames *et al.*, 1975). These *Salmonella* strains are consequently incapable of growing in a medium lacking histidine. Upon exposure of the organisms to a mutagen, the bacteria may revert to the *his*⁺ genotype and are subsequently capable of growing in the nutrient-deficient medium. The number of *his*⁺ colonies formed is proportional to the number of revertants and hence is indicative of the mutagenic potency of the respective mutagen tested.

While initial studies employing the Ames test indicated only slight or no mutagenic responses to DNP when tester strains TA1535 and TA1537 were employed (Rosenkranz and Mermelstein, 1985), introduction of the plasmid *pKM101*, which codes for error-prone DNA repair enzymes, revealed the extraordinarily potent bacterial mutagenicity of DNP (Mermelstein *et al.*, 1981; Rosenkranz and Mermelstein, 1985; see also Table 1.1).

With the possible exception of the aflatoxins (fungal toxins, e.g. A₁, B₁), DNP are probably the most potent bacterial mutagens known (Rosenkranz and Mermelstein, 1983; 1985). As opposed to DNP however, aflatoxins require the presence of liver microsomal or post-9000 x g supernatant (S9) enzymes to express their mutagenicity in *S. typhimurium*.

A variety of Ames *Salmonella* strains have been developed which allow for the differentiation and characterization of mutagenic expression. These strains display preferential susceptibility to either frameshift mutagens, base-pair substitutions or oxidative damage (i.e., strains TA98, TA100 and TA102, respectively). Furthermore, several *S. typhimurium* strains which are deficient in certain of the enzymes involved in

the metabolic activation of nitroarenes have been selected from these parent strains and have proven to be especially useful in studies concerned with the bacterial activation of DNP. Of these, *S. typhimurium* TA98NR and TA100NR have been selected based on resistance to nitrofurantoin toxicity and have been shown to be deficient in the so-called "classical nitroreductase" (Rosenkranz and Speck, 1975; 1976). The gene encoding this bacterial enzyme which catalyzes the reduction of certain nitroarenes has recently been cloned and sequenced (Watanabe *et al.*, 1990). As DNP have been found to be especially mutagenic in tester strains specific for frameshift mutagens i.e., the mutagenic response to DNP is approximately one order of magnitude greater in TA98 than in TA100 (Table 1.1), the remainder of this discussion will focus on this strain and its respective derivatives. Irrespective of the tester strain employed, the order of mutagenic potency is always 1,8- > 1,6- > 1,3-DNP.

The mutagenicity of 1-nitropyrene (1-NP) and 1,3-DNP was found to be greatly reduced in TA98NR compared to TA98. The mutagenicities of 1,6- and 1,8-DNP on the other hand were not affected or only slightly affected (Table 1.1). Differences in the mutagenic response of TA98 vs. TA98NR are not only limited to nitrated pyrenes. For example, the mutagenicity of 2-nitrofluorene and the nitrofuran nifurtimox was strongly reduced while that of the nitroazaarene 4-nitroquinoline N-oxide was not altered (Rosenkranz *et al.*, 1987). These apparent substrate specificities were the basis for the realization that other nitroreductases must exist in *Salmonella*, i.e. (an) enzyme(s) which recognize(s) 1,6- and 1,8-DNP (Howard *et al.*, 1987a).

A further *Salmonella* mutant derivative of TA98 is *S. typhimurium* TA98/1,8-DNP₆. This strain was selected because of its insensitivity to 1,8-DNP. While it was initially thought that this strain is deficient in a nitroreductase activity which recognizes 1,8-DNP (Rosenkranz *et al.*, 1982), subsequent studies have shown that 1-nitro-8-

nitrosopyrene does not display equivalent mutagenicity in TA98/1,8-DNP₆ and TA98 (Fifer *et al.*, 1986). As metabolism of 1-nitro-8-nitrosopyrene does not require nitroreduction, lack of equivalent mutagenicity would not have been expected if the enzyme lacking was indeed a nitroreductase. Similar observations that exclude the possibility of this *Salmonella* strain being deficient in a nitroreductase enzyme were made using the toxicants 1-nitrosopyrene and 2-nitrosofluorene (Rosenkranz and Mermelstein, 1985). Thorough studies by McCoy *et al.* (1982; 1983) involving a series of 2-nitrofluorene metabolites have led to the conclusion that *S. typhimurium* TA98/1,8-DNP₆ is deficient in an arylhydroxylamine esterification enzyme i.e., a bacterial acetylCoA-dependent O-acetyltransferase. That the resistance of TA98/1,8-DNP₆ towards 1,3- and 1,8-DNP mutagenicity was also due to a deficiency in this enzyme was subsequently demonstrated (Orr *et al.*, 1985). Table 1.1. summarizes the mutagenic specificities and potencies of nitrated pyrenes in several *Salmonella* strains incorporating data from a number of laboratories.

Despite the extraordinary mutagenic potency of DNP, a high degree of variability in the mutagenic response to these compounds has been observed between laboratories in which work has been performed under presumably identical conditions. This variability, which appears to be rather typical for nitroarenes has been investigated and reported previously for the nitroarene 2-nitrofluorene (Rosenkranz and Mermelstein, 1985). It seems noteworthy in this regard that 2-nitrofluorene is commonly employed as a reference standard for the mutagenic response of *S. typhimurium* TA98.

Recently, Oda *et al.* (1985) have developed a new system for the detection of mutagens and carcinogens called the *umu*-test. The *umu*-test is based on the bacterial SOS-response upon exposure of bacterial DNA to DNA-damaging agents such as UV-light or certain chemicals. The *umu* operon is under control of the *recA/lexA* system,

TABLE 1.1.

Mutagenicity of nitroarenes and their metabolites in *Salmonella typhimurium* TA100, TA98, TA98NR and TA98/1,8-DNP₆.

mutagen:	revertants/nmol			
	TA100	TA98	TA98NR	TA98/1,8-DNP ₆
1,3-dinitropyrene	42,280 ¹	144,760 ¹	24,750 (17%) ^{a,1}	2750 (2%) ¹
1,6-dinitropyrene	12,159 ¹	183,570 ¹ 255,000 ²	190,900 (104%) ¹ 209,000 (82%) ²	45,890 (25%) ¹ 32,000 (22%) ²
1-nitroso-6-nitropyrene		34,000 (13%) ^{b,2}	33,000 (97%) ²	9,000 (26%) ²
1,8-dinitropyrene	55,420 ¹	254,000 ¹ 157,005 ³ 734,000 ²	264,160 (104%) ¹ - 401,000 (55%) ²	45,890 (25%) ¹ 1,302 (1%) ³ 8,000 (1%) ²
1-nitroso-8-nitropyrene		82,000 (11%) ^{b,2}	103,000 (125%) ²	2,000 (2%) ²

a. Per cent revertants/μg as compared to *S. typhimurium* TA98.

b. Per cent revertants as compared to the parent DNP.

1. Data taken from Rosenkranz *et al.*, 1987

2. Data taken from Fifer *et al.*, 1986.

3. Data taken from Consolo *et al.*, 1989.

which are the regulatory elements of the bacterial SOS-response (Shinagawa *et al.*, 1983). While the function of the *umuDC* gene products has not been clarified to date, this operon is thought to be involved in mutagenesis more directly than other known SOS genes (Kato and Shinoura, 1977) because it is essential for inducible mutagenesis and its products are likely to code for error-prone DNA repair functions (Kato *et al.*, 1982; Shinagawa *et al.*, 1983). A derivative of the Ames tester strain *S. typhimurium* TA1535 (Ames *et al.*, 1975) was constructed by introducing the plasmid *pSK1002*, which carries a *umuC-lacZ* fused gene. The resulting strain *S. typhimurium* TA1535/pSK1002 produces a hybrid protein with β -galactosidase activity. Expression of this chimeric protein is under control of the *umu* regulatory region (Shinagawa *et al.*, 1983). The specific activity of β -galactosidase expressed in *S. typhimurium* TA1535/pSK1002 upon exposure to DNA-damaging agents is hence indicative of their potency to induce *umu* gene induction and therefore reflective of the mutagenic potency of the respective agent.

The sensitivity of the *umu*-test was found to be comparable to that of the Ames *Salmonella* mutagenicity test (Oda *et al.*, 1985). In a subsequent study, 151 chemicals were evaluated for their SOS-inducing activity in the *umu*-test (Nakamura *et al.*, 1987). A comparison of the *umu*-test with the Ames *Salmonella* mutagenicity test revealed that most chemicals found to give a positive response in the Ames test also scored positive in the *umu*-test, underlining the validity of the *umu*-test as a bacterial mutagenicity assay. In comparison to the *umu*-test however, the Ames-test was reported to suffer from certain disadvantages (Oda *et al.*, 1985; Nakamura *et al.*, 1987). Firstly, while the Ames tester strains are specific for certain types of mutations such that several tester strains have to be employed to evaluate the mutagenicity of a certain genotoxin, the *umu*-test is thought to detect either type of mutation with comparative ease. While the

specificity of the Ames tester strains certainly may be an advantage when characterizing the mode of action of any particular mutagen, the potency of a mutagen which may cause more than one type of DNA damage may not be accurately reflected in the Ames test but should be well reflected in the *umu*-test. Another major consideration when employing bacterial mutagenicity tests to study the mutagenic potency of any particular genotoxin is that mutagenicity is often associated with toxicity to the bacterial cells. In the Ames test, the bacterial cells are plated on an Agar plate and exposed to the toxicant for 48 hours. Toxicity to the cell may in some cases lead to erroneous results and is therefore a major concern when conducting this assay. In contrast, the *umu*-test usually requires only two hours of exposure of the bacterial cells to the toxicant in a liquid medium, thereby dramatically reducing the likelihood of interfering toxic effects.

Caution should be exercised when employing either assay system in mutagenicity studies. For 12 out of 151 compounds tested in the *umu*-test contradictory results to the mutagenicity as predicted by the Ames test have been reported (Nakamura *et al.*, 1987). One particularly striking example is the case of sodium azide, which is a potent mutagen in TA100 and is, in fact, used as the positive control mutagen in this strain (Ames *et al.*, 1975, Maron and Ames, 1983). In contrast, sodium azide does not induce SOS-dependent expression of the chimeric *umuC-lacZ* gene. These discrepancies have not been completely resolved however, the mutagenicity of this compound is not enhanced by the plasmid *pKM101*. Sodium azide therefore appears to be one example of a *umuDC*-independent mutagen (Nakamura *et al.*, 1987).

In summary, both mutagenicity assays have advantages and disadvantages which in fact may in some cases complement each other. It is therefore certainly beneficial to compare and contrast results obtained with both assays rather than to rely on the predictive value of either one single assay system. Most importantly, it should be

kept in mind that the two assay systems reflect very different endpoints in the process of mutagenesis. The Ames assay only detects a specific type of mutation which has occurred at a defined, labile region of one particular gene while the *umu*-test indicates a somewhat suicidal response of the cell due to extensive DNA damage, without necessarily reflecting mutation fixation within the bacterium.

1.3.2. Genetic effects, genotoxicity and carcinogenicity of DNP in mammalian cells

The potent bacterial mutagenicity of DNP is paralleled by their genotoxic and carcinogenic effects in mammalian cells (reviewed in Rosenkranz and Mermelstein, 1985). Interestingly, rather remarkable differences are noted in different target cell lines and organs. Mixtures of nitrated pyrenes have been shown to stimulate DNA repair synthesis in HeLa cells (Campbell *et al.*, 1981), a human cell line, and increased frequencies of sister-chromatid exchanges (SCE) in chinese hamster ovary cells have been reported following their exposure to 1,8-DNP (Nachtman and Wolff, 1982). Similarly, chromosomal aberrations in a rat epithelial cell line were observed upon exposure to 1,6- and 1,8-DNP (Rosenkranz and Mermelstein, 1985). Using diphtheria toxin resistance as the phenotypic selection marker in cultured chinese hamster lung cells, Nakayasu and coworkers (1982) demonstrated that all three DNP were mutagenic in this assay system, the order of potency being 1,6- > 1,8- > 1,3-DNP. In newborn mice intraperitoneally (IP) administration of 1,6-DNP produced a statistically significant increase in liver tumors above solvent (dimethylsulfoxide) treated controls (Wislocki *et al.*, 1985). In addition 1,3-, 1,6- and 1,8-DNP have all been shown to induce sarcomas at the site of subcutaneous injection in rats (Ohgaki *et al.*, 1984; Imaida *et al.*, 1986) and

BALB/c mice (Tokiwa *et al.*, 1984). 1,6-DNP has been shown to cause lung cancer in up to 85% of rats following direct injection at this site (Iwagawa *et al.*, 1989).

1.4. Bacterial and mammalian metabolism of DNP

1.4.1. Bacterial metabolism of DNP

The initial step in the bioactivation of DNP is the sequential reduction of one of the two nitro groups to the corresponding nitroso- or hydroxylamino group (McCoy *et al.*, 1981; Rickert, 1985; Rosenkranz *et al.*, 1982; Howard *et al.*, 1987). That this activation step is obligatory in bacteria is evinced by the selection of nitroreductase-deficient *Salmonella* strains (i.e., TA98NR and TA100NR) which display lowered susceptibility to DNP mutagenicity, as discussed above. The requirement for nitroreduction is further supported by the isolation and identification of the DNA-adducts formed upon exposure of bacterial DNA to DNP *in vitro* and *in vivo* (Bryant *et al.*, 1984; Andrews *et al.*, 1986). The primary DNA adduct of 1,8-DNP in *Salmonella* has been identified as 1-N-(2'-deoxyguanosin-8-yl)-amino-8-nitropyrene (McCalla *et al.*, 1985; Andrews *et al.*, 1986), an adduct in which the DNP clearly requires reduction of a nitro moiety.

It has been postulated that the initial nitroreduction step may proceed through sequential one- or two-electron transfers (Howard *et al.*, 1987a). As complete reduction of a nitro group to the corresponding amine involves the total transfer of six electrons, five intermediates are theoretically possible, two of which are the corresponding nitroso- and hydroxylamino compounds and three are free radical species (i.e., the nitro anion radical, the nitroxyl radical and the aminoxyl radical) (see Figure 1.1). Rosenkranz and

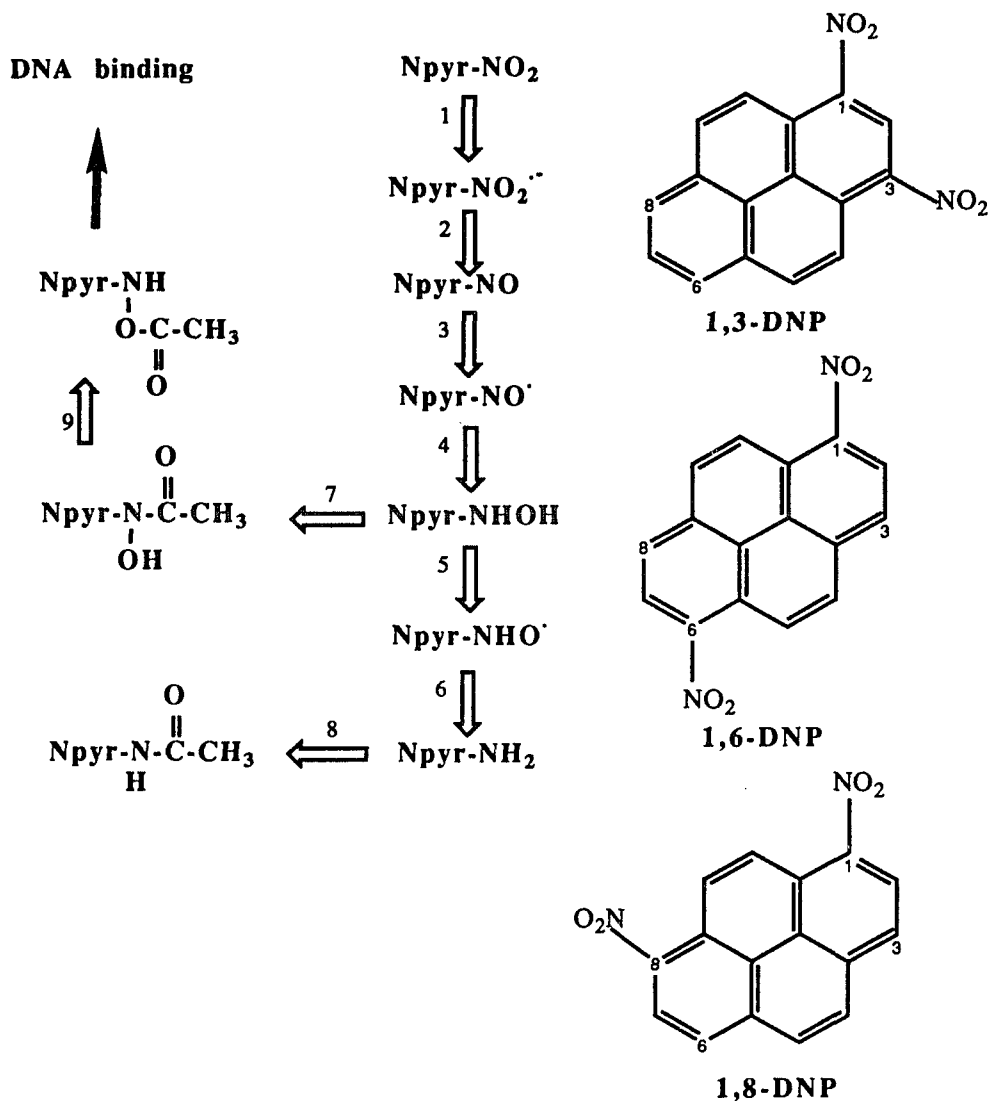


Figure 1.1. Sequential nitroreduction and acetylation of dinitropyrenes. The structures of 1,3-, 1,6- and 1,8-dinitropyrene are shown on the right hand side. Npyr = nitropyrene fragment. Reaction 1-6: Sequential one electron reduction; 7: N-acetylation of hydroxylamino-nitropyrene; 8: N-acetylation of aminonitropyrene 9: N,O-transacetylation of hydroxylamino-nitropyrene.

coworkers have investigated the characteristics of nitroreduction of 1-NP as well as that of 1,3-, 1,6- and 1,8-DNP (Klopman *et al.*, 1984, Eddy *et al.*, 1986). It was found that the first reversible step upon electrochemical nitroreduction of 1-NP and 1,3-DNP corresponds to the transfer of one electron while that of 1,6- and 1,8-DNP corresponds to a divalent reduction (Eddy *et al.*, 1986). This difference in the initial electron-transfer step during the nitroreduction of 1-NP and 1,3-DNP versus that of 1,6-DNP and 1,8-DNP is paralleled by the decreased mutagenicity of 1-NP and 1,3-DNP, but not 1,6- and 1,8-DNP in *S. typhimurium* TA98NR, as compared to TA98. These researchers therefore concluded that the "classical nitroreductase" which is deficient in TA98NR may be a one-electron reductase (Howard *et al.*, 1987a) and inferred that 1,6- and 1,8-DNP must be reduced by other bacterial nitroreductases which preferentially catalyze two-electron reduction of their substrates.

The existence of other nitroreductase enzymes in bacteria is evidenced by the fact that a bacterial nitroreductase has been partially purified which catalyzes the reduction of 4-nitroquinoline N-oxide (Tatsumi *et al.*, 1981), a mutagen whose mutagenic potency is not affected by a deficiency in the "classical nitroreductase" (Rosenkranz *et al.*, 1987). Interestingly, this nitroreductase was found to be inhibited by dicoumarol, a known potent inhibitor of mammalian NAD(P)H-quinone oxidoreductase (DT-diaphorase, [E.C. 1.6.99.2]), which catalyzes the two-electron reduction of its substrates (Prochaska, 1988). These researchers consequently termed this nitroreductase "bacterial DT-diaphorase" (Tatsumi *et al.*, 1981).

Reduction of DNP by bacterial cytosol has been shown to result in the formation of nitrosonitro- and aminonitropyrenes (Djuric *et al.*, 1986; Heflich *et al.*, 1986). These observations imply that hydroxylamino-nitropyrenes must be formed as necessary intermediates. Unfortunately these intermediates are difficult to detect because of their

high reactivity with macromolecules (i.e., DNA or proteins) and molecular oxygen. That these intermediates are indeed capable of redox cycling with molecular oxygen is suggested by the fact that the presence of molecular oxygen in the incubation mixture, as compared to its absence, decreases the formation of aminonitropyrenes in favor of the more oxidized nitrosonitropyrenes, with an overall decrease in the amount of metabolites formed from the parent DNP (Djuric *et al.*, 1986). This decrease in total metabolism may well reflect autooxidation of the nitrosonitropyrene, leading to the recycling of DNP and thus to a limiting of the total steady state level of metabolites formed¹. While the formation of odd-electron reduced radical species of DNP is supported on several grounds i.e., oxygen-sensitivity of several nitroreductases and the electrochemical reduction properties of 1-nitropyrene and 1,3-DNP, no direct experimental evidence for the formation of such radical species has been reported. However, nitro anion radicals of a wide variety of nitroarenes including nitrofurantoin, formed upon enzymatic reduction of the parent compound by microsomes or purified enzymes, have been detected by electron spin resonance techniques (Mason, 1982).

Despite their intrinsic instability, there is further indirect evidence for the formation of hydroxylaminonitropyrenes. As discussed above, the mutagenicity of 1,3- and 1,8-DNP is virtually abolished in *S. typhimurium* TA98/1,8-DNP₆, a strain deficient in AcCoA-dependent O-acetyltransferase. The only reductive metabolite of DNP which can conceivably be O-acetylated is the hydroxylamine, leading to the

¹ This mechanism is generally referred to in the literature as inhibition of nitroreductase enzymes by oxygen. Since the presence of oxygen decreases the stability of the products formed, but does not necessarily interact with the enzyme itself, this term is somewhat misleading. In the case of xanthine oxidase, which has been shown to catalyze the reduction of 1-nitropyrene to 1-hydroxylaminopyrene (Howard and Beland, 1982), two modes of inhibition by oxygen are feasible. Firstly, the amount of reduced metabolites formed may be decreased by redox cycling. Secondly, oxygen is a known electron acceptor for the molybdoflavoenzyme and therefore oxygen may compete with the nitro substrate for reducing equivalents at the active site of the enzyme.

formation of highly reactive N-acetoxyaminonitropyrenes. N-Acetylation may occur with hydroxylamino- and aminonitropyrenes as well as the completely reduced diaminopyrenes, all of which are thought to be far less reactive towards DNA than the O-acetylated metabolites. Spontaneous hydrolysis of acetoxyaminonitropyrene putatively leads to the formation of the highly electrophilic nitrenium ion, which can react directly with DNA. It is this pathway whose efficacy is decreased in *S. typhimurium* TA98/1,8-DNP₆ due to decreased formation of the O-acetylated metabolite, as shown by a greatly diminished formation of acetoxyaminofluorene from hydroxylaminofluorene (McCoy *et al.*, 1983). The lack of mutagenic response to 1,3- and 1,8-DNP in the acetylase-deficient strain TA98/1,8-DNP₆ is possibly the strongest evidence for the formation of hydroxylaminonitropyrenes.

1.4.2. Metabolism of DNP by mammalian cell fractions

Metabolism of DNP by mammalian cell fractions shows striking similarities to their metabolism in bacteria in that nitroreduction and, at least in some instances, acetylation are required for activation of DNP to DNA-reactive electrophiles. The liver cytosolic enzymes xanthine oxidase, aldehyde oxidase and NAD(P)H-quinone oxidoreductase have been suggested to catalyze nitroreduction of DNP (Djuric *et al.*, 1986; 1988; Beland and Kadlubar, 1990). Purified xanthine oxidase has been shown to efficiently reduce 1-NP under anaerobic conditions and to catalyze the formation of N1-(5'deoxyguanosin-8-yl)-aminopyrene in the presence of DNA (Howard and Beland, 1982). A recent report (Djuric and McGunagle, 1989) shows that incubation of radiolabelled 1,6-DNP with xanthine oxidase under anaerobic conditions leads to the association of radioactivity with DNA, although it appears the enzyme is much less

efficient in catalyzing the reduction of dinitropyrenes than 1-nitropyrene (Bauer and Howard, 1990). Addition of acetylCoA to rat liver cytosolic preparations strongly enhances the DNA-binding capacity of 1,6-DNP and concomitantly increases the formation of acetylated metabolites, suggesting that acetylase activities towards DNP are not unique to bacteria (Djuric *et al.*, 1985). Furthermore this observation is the basis for the assumption that the amount of N-acetylated metabolites of DNP are indicative of the amount of DNA-reactive electrophile formed (Heflich *et al.*, 1986).

Microsomal nitroreduction of DNP has been proposed to be catalyzed by NADPH-cytochrome P450 reductase (Djuric *et al.*, 1988) and it has recently been reported that purified rat liver NADPH-cytochrome P450 reductase decreases the bacterial genotoxicity of 1,6- and 1,8-, but not 1,3-DNP in the *umu* gene induction assay (Shimada and Guengrich, 1990).

Both the *umu* genotoxicity and Ames mutagenicity assays have been employed to study the effect of mammalian cell fractions on the bacterial mutagenicity of DNP. Addition of a 9000 x g supernatant (S9) results in a detoxification of DNP, either by metabolism of the parent DNP or of the intermediates produced by the *Salmonella* strains, to less reactive metabolites (Rosenkranz and Mermelstein, 1983; Tokiwa *et al.*, 1981; Traynor *et al.*, 1988; Shah *et al.*, 1990). Tokiwa *et al.* (1981) reported that the presence of S9 from Aroclor-pretreated rats almost completely abolished the bacterial mutagenicity of 1,6- and 1,8-DNP. The 1,6-DNP isomer was found to be more refractory to detoxification than was 1,8-DNP and the deactivating capacity of S9 fractions was far more pronounced in tester strain *S. typhimurium* TA98 than in *S. typhimurium* TA100. This potent deactivation of DNP by S9 in the Ames test has also been reported by other investigators (Mermelstein *et al.*, 1981; Rosenkranz and

Mermelstein, 1985), who found, that the enzyme fraction responsible for deactivation requires NADPH and is located in the microsomal fraction.

Traynor *et al.* (1988) reported on the modulation by chronic ethanol feeding and Aroclor-pretreatment of the *in vitro* mutagenicity of three dinitropyrene isomers (1,3-, 1,6- and 1,8-DNP). The mutagenicity of the DNP isomers toward *S. typhimurium* TA98 and TA100 was attenuated in the presence of post-mitochondrial supernatants (S9) from both ethanol-fed and pair-fed rats albeit, that from the ethanol-fed group was more efficient in lowering the mutagenicity. Pretreatment with Aroclor-1254 resulted in >98% detoxification by S9 of all three DNP. A higher protein concentration was required to detoxify the DNP isomers when TA100 was employed as compared to TA98. The cytosolic fraction from ethanol-fed rats enhanced the mutagenicity of all of the DNP isomers in TA100. The most notable enhancement was with 1,3-DNP in which case the mutagenicity was more than 4-fold higher in the presence of cytosol. Cytosol from pair-fed rats enhanced only the mutagenicity of 1,3-DNP, this by almost 3-fold. In contrast, cytosol from Aroclor-pretreated animals was significantly less activating than was that of the pair-fed control system. Microsomes decreased the mutagenicity of DNP similarly to S9 i.e., fractions from ethanol-fed and Aroclor-pretreated rats were more efficient than those of pair-fed rats in deactivating all the DNP isomers. However, per mg of protein, detoxification of DNP by S9 was more efficient than with microsomes thus, cytosolic and microsomal enzymes are both required for maximal detoxification. The authors concluded that S9 contains two balancing activities, a cytosolic activity that augments and a microsomal activity that negates the bacterial mutagenicity of DNP, both of which are modified by chronic ethanol feeding or Aroclor-1254-pretreatment (Traynor *et al.*, 1988; Winston *et al.*, 1991).

Shah *et al.* (1990) also investigated the effect of cytosol, microsomes and S9 on the bacterial mutagenicity of 1,8-DNP. The study further evaluated the effect of Aroclor-1254-pretreatment of experimental animals on the modulation of 1,8-DNP mutagenicity by these three subcellular fractions. Consistent with previous reports (Tokiwa *et al.*, 1981; Traynor *et al.*, 1988), liver S9 from untreated rats was found to decrease the mutagenicity of 1,8-DNP towards TA98 over the entire range of mutagen concentrations tested (0.1-33.3 ng/plate) and pretreatment of rats with Aroclor-1254 dramatically potentiated this effect. Microsomes from either source displayed virtually the same effects as the corresponding S9 fraction. Cytosol was found to be a potent activator of 1,8-DNP; it increased the number of TA98 revertants scored in the presence of 1,8-DNP by 2.5-3.5-fold. As observed by Traynor *et al.* (1988), cytosol from Aroclor-1254-pretreated rats was found to be substantially less activating than cytosol from the corresponding control group. It was concluded that Aroclor-1254 pretreatment induced microsomal factors which block the mutagenicity of 1,8-DNP and suggested further, that the nitroreductase activity located in the soluble fraction (cytosol) was unlikely to be responsible for the cytosolic augmentation of 1,8-DNP mutagenicity.

1.5. Hepatic ethanol toxicity and the effect of ethanol consumption on the hepatic metabolism of xenobiotic compounds

Ethanol ingestion causes a wide variety of enzymatic and metabolic changes, either directly through the metabolism of ethanol itself or as a consequence of ethanol metabolism mediated by specific alterations such as cofactor availability or enzyme induction (reviewed in Lieber, 1990; Videla and Valenzuela, 1982). Amongst these alterations are a decreased NAD⁺/NADH-ratio (Khanna *et al.*, 1969), a decrease in the

hepatic ratio of reduced versus oxidized glutathione (Videla and Valenzuela, 1982) and induction of cytosolic glutathione-S-transferases (Hetu *et al.*, 1986). Further, cytosolic alcohol dehydrogenase has been reported in some instances to be induced upon ethanol ingestion (reviewed in Khanna and Israel, 1980), and the ratio of xanthine dehydrogenase to xanthine oxidase is decreased. Finally, an overall induction of the total xanthine dehydrogenase/xanthine oxidase activity is seen (Abbondanza *et al.*, 1989).

Ethanol may be oxidized by cytosolic alcohol dehydrogenases or by peroxisomal catalase, albeit the contribution of this latter enzyme activity to the *in vivo* metabolism of ethanol is thought to be minor (Oshino *et al.*, 1975). Another locus of ethanol oxidation is the microsomal ethanol oxidizing system (MEOS), an enzymatic activity associated with the microsomal mixed function oxidase system (Koop and Coon, 1986; Teschke and Gelbert, 1986). Studies by Koop *et al.* (1982) and Ryan *et al.* (1985; 1986) have revealed the existence of an ethanol-inducible cytochrome P450 in rat (cytochrome P450j) and rabbit (cytochrome P450 LM3a), all of which are referred to as CYP 2E1¹ according to the unifying nomenclature proposed by Nebert *et al.* (1989). The enzyme has been purified from both rabbit and rat liver microsomes (Koop *et al.*, 1982; Ingelman-Sundberg and Johansson, 1984; Ryan *et al.*, 1985; 1986).

Cytochrome P450 2E1 is not only highly inducible by ethanol but also by acetone, isoniazid (Ryan *et al.*, 1985; 1986) and pyrazole (Tu *et al.*, 1981). The enzyme not only catalyzes the oxidation of ethanol to acetaldehyde, but also the selective oxidation of higher aliphatic alcohols such as 1-butanol and 1-pentanol (Morgan *et al.*,

¹ Recent evidence suggests the existence of two genes encoding for ethanol-inducible cytochrome-P450 enzymes, namely CYP 2E1 and CYP 2E2. The latter isozyme has very recently been isolated and characterized from rabbit liver and is virtually identical to CYP 2E1 in its catalytic, structural and immunological characteristics.

1982) and of the solvents acetone (Koop and Casazza, 1985) and carbon tetrachloride (Johanson and Ingelman-Sundberg, 1985), amongst others. This P450 isoform displays specificity towards the the ring-hydroxylation of benzene (Johanson and Ingelman-Sundberg, 1988), aniline (Morgan *et al.*, 1982; Ko *et al.*, 1987; Winston and Narayan, 1988) and p-nitrophenol (Koop, 1986) as well as the demethylation of N-nitrosodimethylamine (Peng *et al.*, 1982; Yang *et al.*, 1985). Because of the strong association between chronic ethanol consumption and the development of certain types of cancer (Lieber *et al.*, 1979), the effects of ethanol-inducible cytochrome P450 on the metabolism of potentially mutagenic xenobiotic compounds has received widespread attention, such as with nitroarenes (Howard *et al.*, 1987b; 1988; Traynor *et al.*, 1988), arylamines (Steele and Ioannides, 1986, Traynor *et al.*, 1991), polycyclic aromatic hydrocarbons (Seitz *et al.*, 1978) and nitrosamines (McCoy *et al.*, 1979; Garro *et al.*, 1981; Glatt *et al.*, 1981; Sohn *et al.*, 1987, Ko *et al.*, 1987).

There are a variety of determinants associated with ethanol consumption which may alter the metabolic fate of xenobiotics in general and that of nitroarenes in particular. At one hand there is the aforementioned induction of the ethanol-inducible cytochrome P450 (CYP 2E1) which selectively catalyzes the hydroxylation of p-nitrophenol. On the other hand ethanol consumption has been reported to increase cellular concentrations of acetylCoA (Bode *et al.*, 1970; Kondrup *et al.*, 1973), which is the cofactor for N- and O-acetyltransferases. This increase in cofactor availability may thus result in an increased rate of drug acetylation if the concentration of cofactor is rate-limiting. Whether or not ethanol consumption alters cytosolic N-acetyltransferase activities has not been investigated and data pertaining to this subject will be presented in this work.

Chapter 2

Ethanol-induced enzymatic alterations

2.1. Introduction

Alterations in the hepatotoxicity and mutagenicity of xenobiotics upon chronic ethanol consumption may be, in part, a consequence of enhanced microsomal activation of drugs by the microsomal cytochrome P450 system (as discussed in chapter 1). Cytosolic oxidative or reductive metabolism of toxicants may also be involved however. In addition, interference of the conjugation of xenobiotics and/or their metabolites to glutathione (GSH) as a consequence to chronic ethanol consumption may also alter their metabolic fate (see Videla and Valenzuela, 1982). Liver glutathione peroxidase and glutathione reductase activities have been found to increase by 45% and 15% respectively, after chronic ethanol intake (Teschke *et al.*, 1977). These two enzymes play an important role in the regulation of the oxidized versus reduced glutathione ratio and their increase upon ethanol consumption is thought to be an adaptive response to the increased demand for reduced GSH in the liver due to ethanol-exacerbated oxidative tissue damage (Videla and Valenzuela, 1982).

Glutathione-S-transferase (GST) is a cytosolic phase II enzyme which catalyzes the conjugation of a wide variety of substrates with GSH¹. A number of GST isoenzymes with differing, yet overlapping substrate specificities are present in rodent liver (reviewed by Habig and Jakoby, 1981). Hetu *et al.* (1986) previously reported that GST-activity was slightly increased in response to ethanol ingestion. As shown by Asokan and Takahashi (1983; 1989) in monkey liver, GST can catalyze the conjugation of GSH to several nitroarenes including dinitrobenzenes. This conjugation step is accompanied by the loss of the nitro moiety. In light of the fact that the nitro group is essential for the toxic and mutagenic effects mediated by nitroarenes and the possible induction of this GST activity upon chronic ethanol, it was of interest to investigate the role of GST in the ethanol-modulated metabolism of nitroarenes.

Another cytosolic conjugation reaction involves the N- or O-acetylation of amines or hydroxylamines formed upon reductive metabolism of nitroarenes (see section 1.4.). The latter may also be formed by microsomal mixed-function oxidase- (Masson *et al.*, 1983; Garner *et al.*, 1984) and FAD monooxygenase-mediated (Pelroy and Gandolfi, 1980; Hammons *et al.*, 1985) N-hydroxylation of arylamines. Hepatic cytosol contains enzymes which can N-acetylate the amine functional group or N,O-transacetylate the N-acylated arylhydroxylamines (Lower and Bryan, 1973). The role of ethanol as a modifier of arylamine acetylation/deacetylation reactions by both cytosolic and microsomal enzymes has not been investigated concomitantly. Because it has been hypothesized that acetylation of the hydroxylamine forms the ultimate mutagen (Miller

¹ The term conjugation refers to the phase II metabolism of xenobiotic substances which are commonly first metabolized by the microsomal mixed function oxidase or phase I enzyme system (i.e., cytochrome P450). The phase I system catalyzes redox reactions which usually provide an anchor group e.g. ring hydroxylation of a polyaromatic hydrocarbon (PAH). This hydroxylation reaction facilitates phase II metabolism, that is the conjugation of the hydrophobic substrate with a hydrophilic endogenous compound (glutathione, sulfate, UDP-glucose, acetate). This more hydrophilic adduct may be readily excreted via the hepatobiliary system.

and Miller, 1983; McCoy *et al.*, 1982; 1983), modulation of these activities by ethanol consumption could be a critical step in ethanol-mediated alteration of nitroarene and arylamine mutagenesis.

The current chapter (a) validates the appropriateness of the experimental system employed to study the effects of chronic ethanol ingestion; (b) indicates marker activities altered as a consequence of chronic ethanol consumption; and (c) elucidates ethanol-induced alterations which may be involved in the metabolism of nitroarenes. The data should help to target mechanisms by which ethanol consumption may affect the metabolism and mutagenicity of nitroarenes, in particular that of DNP.

2.2. Materials and methods

2.2.1. Materials: Pyrazole, 3-methylcholanthrene, bovine serum albumin, p-nitrophenol, 4-nitroquinoline-N-oxide, NADP⁺, NADH, Folin-Ciocalteu reagent, magnesium chloride, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, potassium ferricyanide, potassium phosphate, sucrose, glutathione, EDTA, desferrioxamine, acetylCoA, acetylphosphate and phosphotransacetylase were from Sigma Chemical Co., St. Louis, MO. 2-Acetylaminofluorene, 2-aminofluorene, 1-chloro-2,4-dinitrobenzene and 4-nitrocatechol were from Aldrich, Milwaukee, WI and Aroclor from Foxboro Analabs, North Haven, CT. All other chemicals were of the highest purity grade commercially available.

2.2.2. Animals: maintenance and pretreatment: Male Sprague-Dawley rats were purchased from Hilltop Lab Animals, Inc., Scottsdale, PA and rats were housed individually in stainless steel, wire cages. A 12:12 hour light : dark cycle was

maintained throughout the regimen. Animals were acclimated for 1 week, during which they were maintained on Purina rat chow and water *ad libitum*.

2.2.2.1. Ethanol-feeding: Following acclimation, rats (150-200 g) were weight-paired and maintained for 30 days on a liquid diet containing 36% of the total calories as ethanol (Lieber and DiCarli, 1982). Controls were isocalorically pair-fed carbohydrate in place of ethanol.

2.2.2.2. Pretreatment of animals with Aroclor-1254, pyrazole and 3-methylcholanthrene: Aroclor-1254 (500 mg/kg body weight) was administered intraperitoneally (i.p.) as a single injection 4 days prior to sacrifice. 3-Methylcholanthrene (3-MC, 35 mg/kg body weight in corn oil) and pyrazole (200 mg/kg body weight in saline) were administered i.p. on days 1,3 and 4 prior to sacrifice on day 5.

2.2.2.3. Tissue preparation: Animals were killed by decapitation. The livers were quickly excised and weighed and then washed and homogenized in ice-cold 10 mM Tris pH 7.4 containing 0.25 M sucrose and 1 mM EDTA (STE-buffer) using a Potter-Elvehjem teflon homogenizer. A 1:5 ratio (w/v) of tissue to buffer was used in all cases (Winston and Narayan, 1988). Differential centrifugation of the homogenate was used to produce fractions termed S9, cytosol and microsomes. Following two initial spins of 500 x g and 6000 x g for 10 mins each, a subsequent spin of 9,000 x g for 10 mins produced a post-mitochondrial supernatant (S9). A further centrifugation of the S9 fraction at 105,000 x g for 60 mins produced the cytosolic fraction (supernatant) and the microsomal fraction (pellet). The latter was washed by resuspending in 0.125 M KCl

and recentrifuging for a further 60 mins at 105,000 x g. The supernatant was discarded and the pellet resuspended in 0.125 M KCl. All centrifugation steps were performed at 4°C, and all fractions were stored at -70°C until required for use. The protein content of each fraction was determined using either the method of Lowry *et al.* (1951) or Bradford *et al.* (1978). Bovine serum albumin was used for standard protein concentrations in each case.

2.2.3. Enzyme assays

2.2.3.1. Determination of the microsomal contents of cytochrome P450, P420 and b₅, and the activity of NADH-dependent reductase: Microsomal cytochrome P450 and P420 contents were determined in 0.1 M potassium phosphate buffer pH 7.4 containing 20% (v/v) glycerol from the carbon monoxide-difference spectrum method of Omura and Sato (1964) as modified by Estabrook and Werringloer (1978). Extinction coefficients of $E_{450\text{nm}} = 91 \text{ mM}^{-1} \times \text{cm}^{-1}$ and $E_{420\text{nm}} = 110 \text{ mM}^{-1} \times \text{cm}^{-1}$ were used in each case. Cytochrome b₅ content was determined in the same buffer by the method of Estabrook and Werringloer (1978). An extinction coefficient of $E_{426\text{nm}-409\text{nm}} = 185 \text{ mM}^{-1} \times \text{cm}^{-1}$ was used for quantification of cytochrome b₅ (Estabrook and Werringloer, 1978)

NADH-ferricyanide reductase activity was determined with 0.1-0.2 mg microsomal protein in 0.1 M potassium phosphate buffer pH 7.4 containing 20% (v/v) glycerol as described by Ichikawa *et al.* (1969). 2 mM Potassium ferricyanide was used as substrate and the reaction started with 200 μM NADH. Activity was determined from the change in absorbance at 420 nm over time using an extinction coefficient of $E_{420\text{nm}} = 1.02 \text{ mM}^{-1} \times \text{cm}^{-1}$ (Comai and Gaylor, 1973).

2.2.3.2. Microsomal hydroxylation of *p*-nitrophenol: Microsomal hydroxylation of *p*-nitrophenol was determined essentially as described by Koop (1986). Microsomes were incubated at 37°C in a final volume of 1 ml in the presence of 200 µM *p*-nitrophenol in 50 mM Tris-HCl, pH 7.4. The reaction was initiated by addition of an NADPH-generating system consisting of 5 mM glucose-6-phosphate, 2.5 mM NADP⁺, 2.5 mM MgCl₂ and 2 units/ml of glucose-6-phosphate dehydrogenase. Unless otherwise indicated, the reaction was terminated after 10 minutes by the addition of 500 µl of 0.6 M perchloric acid. This was centrifuged at approximately 2500 x g for 5 minutes and then 1 ml of clear supernatant was mixed with 100 µl 10 N NaOH. This was recentrifuged at approximately 2500 x g for 5 minutes and then the absorbance determined spectrophotometrically at 546 nm. An extinction coefficient of $E_{546\text{ nm}} = 9.53\text{ mM}^{-1} \times \text{cm}^{-1}$ was used for calculation of activity. Additional experiments employed carbon monoxide, ethanol (2% (v/v)), DMSO (2% (v/v)) and desferrioxamine (500 µM) as inhibitors.

2.2.3.3. Cytosolic glutathione-S-transferase activities in ethanol- vs. pair-fed rats: Cytosolic glutathione-S-transferase activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was determined according to Habig and Jakoby (1981) in liver cytosol from ethanol- and pair-fed rats. Cytosolic protein (0.025-0.05 mg protein/ml) was incubated in the presence of 1 mM CDNB and 5 mM glutathione (GSH) in 80 mM potassium phosphate buffer, pH 7.5 in a final volume of 1 ml. The change in absorbance due to formation of the GSH-conjugate with CDNB was followed at 345 nm for two minutes. Under these conditions the reaction was linear with respect to protein and time. An extinction coefficient of $E_{345\text{ nm}} = 9.6\text{ mM}^{-1} \times \text{cm}^{-1}$ was used for calculation of GST

activity (Habig and Jacoby, 1981). Glutathione-S-transferase catalyzed conjugation of GSH with 4-nitroquinoline N-oxide (NQO) was determined essentially as outlined by Asoka and Takahashi (1983; 1989).

2.2.3.4. Assay for cytosolic N-acetyltransferase and microsomal deacetylase: Cytosolic acetyl CoA-dependent acetylase activity was measured using the substrates isoniazid and 2-acetylaminofluorene (2-AF). Isoniazid-N-acetyltransferase activity was determined according to Weber (1971). The final reaction mixture contained in 1 ml, 0.6 mM potassium borate buffer, pH 9.0, 0.25 mM acetyl CoA and 1 mM isoniazid. The reaction was initiated by the addition of cytosol and was linear for at least 10 minutes. N-Acetyltransferase activity towards 2-AF was measured according to the procedure of Andres *et al.* (1985) which employs an acetyl CoA-regenerating system (acetylphosphate plus phosphotransacetylase and acetyl CoA). 2-AF was dissolved in methanol to give a final concentration of 0.15 mM. The buffer contained 5% (v/v) DMSO to ensure solubility of the arylamine. Under these conditions, the assay was linear for at least 30 minutes.

Microsomal deacetylation of 2-AAF was assayed essentially as described by Schut *et al.* (1978). The final reaction volume of 1 ml contained approximately 1 mg microsomal protein, 0.25 mM 2-AAF and 140 mM potassium phosphate buffer, pH 7.0. The use of Tris-buffer as indicated by Schut *et al.* (1978) interfered strongly with the formation of the chromophore. The reaction was initiated by addition of substrate, incubated for 30 minutes at 37°C and stopped by addition of 500 µl of 17 % trichloroacetic acid (TCA). A 1.5 ml aliquot of a 1% ethanolic solution of *p*-dimethylaminobenzaldehyde was then added and the color allowed to develop for 30 minutes. The mixture was centrifuged at approximately 2,500 x g for 5 minutes and the

absorbance of the clear supernatant read at 460 nm against a reagent blank. The amount of 2-AF formed was quantified by comparison to a standard curve of known concentrations of 2-AF.

2.3. Results

2.3.1. Effects of chronic ethanol-consumption on the contents of microsomal cytochrome P450- and cytochrome b₅ and the activity of NADH-ferricyanide reductase.

Table 2.1. summarizes the effect of chronic ethanol consumption on three microsomal marker activities. Ethanol ingestion increased the cytochrome P450 specific content by approximately 50% ($p < 0.05$). Cytochrome b₅-content and NADH-dependent ferricyanide reductase activity were also slightly induced ($p < 0.1$).

2.3.2. Hydroxylation of p-nitrophenol by microsomal cytochrome P450.

2.3.2.1. Characterization of the assay:

Dependence on protein concentration and time. Rat liver microsomes from pyrazole pretreated animals were pooled ($n=4$) and used for the characterization of p-nitrophenol (pNP) hydroxylation. The results are shown in Figures 2.1 and 2.2. Linearity of the reaction for at least 30 minutes was observed with protein concentrations up to 0.5 mg microsomal protein/ml incubation mixture. From these observations, a protein concentration of 0.5 mg and a incubation time of 10 minutes was used for subsequent experiments. The UV/VIS-spectrum of the ring-hydroxylated product 4-nitrocatechol was identical to that of a synthetic standard (not shown).

Table 2.1. Effect of chronic ethanol consumption on microsomal P450 and b₅ contents and NADH-ferricyanide reductase activity

	P450 specific content (nmol P450/mg protein)	b ₅ specific content (nmol b ₅ /mg protein)	NADH-ferricyanide reductase (μ mol/min/mg protein)
Feeding regimen:			
Pair-fed	1.0 \pm 0.2	0.4 \pm 0.1	2.0 \pm 0.3
Ethanol-fed	1.6 \pm 0.3 *	0.5 \pm 0.1	2.2 \pm 0.2 **

^a Results are presented as means \pm SD of triplicate determinations of 4 individual animals in each treatment group.

^b Results are at least duplicate determinations or higher if the experimental error exceeded 5% for each individual animal. The mean \pm SD of 4 individual animals for each treatment group is given.

* Significantly different from pair-fed control ($p < 0.05$).

** Significantly different from pair-fed control ($p < 0.1$).

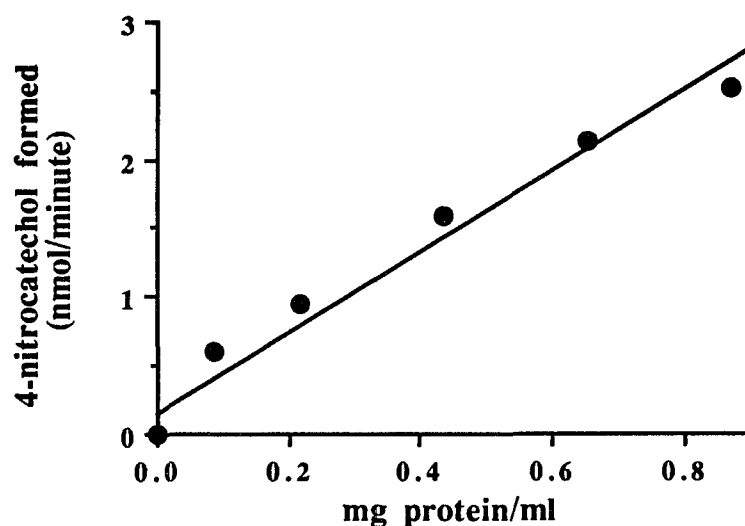


Figure 2.1. Microsomal hydroxylation of p-nitrophenol: Effect of protein concentration. Increasing concentrations of microsomal protein from pyrazole-pretreated rats were incubated for 10 minutes at 37°C in the presence of the substrate pNP and a NADPH-generating system as described in Materials and Methods. Rates are expressed as nmol 4-nitrocatechol formed/minute and results are the mean of duplicate determinations at each protein concentration.

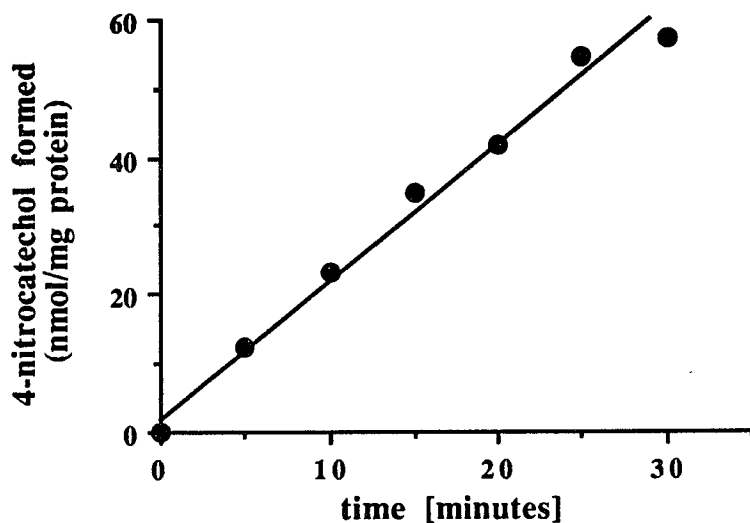


Figure 2.2. Microsomal hydroxylation of p-nitrophenol: Time dependence. Microsomes from pyrazole-pretreated animals (0.5 mg protein/ml) were incubated for up to 30 minutes in the presence of pNP and a NADPH-generating system. Samples were assayed for formation of 4-nitrocatechol as described in Materials and Methods. Rates are expressed as nmol 4-nitrocatechol formed/mg microsomal protein and results are the mean of duplicate determinations at each time point.

2.3.2.2. Hydroxylation of *p*-nitrophenol by microsomal cytochrome P-450: Effect of chronic ethanol ingestion and pyrazole pretreatment:

As discussed in chapter 1, ethanol-inducible cytochrome P450 catalyzes a variety of reactions including ring-hydroxylations of aniline and pNP. Since this study was concerned with the effect of chronic ethanol consumption on the metabolism of nitroaromatics, the hydroxylation of pNP was chosen as a marker activity to determine the extent of induction of CYP2E1 (Table 2.2). The specific activity observed in pyrazole-pretreated microsomes, another inducer of CYP2E1, was also determined. As pyrazole was administered in saline, a saline-pretreated control was included. Rates of aniline hydroxylation have been reported previously by this and other laboratories to be increased with microsomes from ethanol-fed animals compared to pair-fed control animals (Winston and Narayan, 1988; Morgan *et al.*, 1982).

Table 2.2. Microsomal hydroxylation of *p*-nitrophenol: Effect of ethanol consumption and pyrazole pretreatment

Treatment:	nmol 4-nitrocatechol formed/min/mg protein	
Pair-fed	0.7 ± 0.1 ^a	(n=6)
Ethanol-fed	3.0 ± 1.3*	(n=6)
Saline control	1.3 ± 0.3	(n=3)
Pyrazole pretreated	3.8 ± 0.9**	(n=4)

^a Results are presented as mean ± SD of duplicate determinations of the indicated number of samples

* Significantly different from pair-fed control (p<0.01, paired t-test).

** Significantly different from saline control (p<0.01,unpaired t-test).

Increases of pNP hydroxylase of respectively 4.6-fold and 3-fold were observed above pair-fed and saline controls for the effect of chronic ethanol consumption and pyrazole pretreatment. The results are comparable to values reported in the literature (Koop, 1986) and establish the potent effect of both ethanol and pyrazole on the induction of CYP2E1 in the rat.

2.3.2.3. Effects of ethanol, dimethylsulfoxide, carbon monoxide and desferrioxamine on the microsomal hydroxylation of p-nitrophenol:

Microsomes from one experimental animal fed an ethanol-containing diet for 30 days and which displayed high pNP hydroxylase activity were incubated in the presence or absence of 500 μ M desferrioxamine with either carbon monoxide or the CYP2E1 substrates ethanol (1% v/v) or dimethylsulfoxide (2% v/v) (Morgan *et al.*, 1982). The nondiscriminating cytochrome P450 inhibitor carbon monoxide was included as a positive control. Incubations containing desferrioxamine were performed in order to test for the possible involvement of oxygen-derived free radicals e. g., hydroxyl radicals in the microsomal hydroxylation of pNP. Hydroxyl radicals may be formed from Fenton-type reactions which involve iron-catalyzed dismutation of hydrogen peroxide. Microsomal formation of hydroxyl radicals has been reported to be completely inhibited by the presence of the iron chelator desferrioxamine (Winston and Cederbaum, 1982). Table 2.3 summarizes the effects of P450 inhibitors and desferrioxamine on microsomal pNP hydroxylase activity.

Table 2.3. Effects of ethanol, dimethylsulfoxide, carbon monoxide and desferrioxamine on the microsomal hydroxylation of p-nitrophenol

nmol 4-nitrocatechol formed/min/mg protein				
Additive:	no desferrioxamine		with desferrioxamine	
microsomes	4.38, 4.27 ^a	-	4.54, 4.16	(0%)
+ carbon monoxide	1.82, 1.45	(62%) ^b	2.56, 2.72	(40%)
+ ethanol	1.17, 1.07	(74%)	1.34, 1.34	(69%)
+ DMSO	0.48, 0.48	(89%)	0.64, 0.64	(85%)

^a Results of duplicate determinations are shown. Microsomal protein (0.2 mg) was incubated with 200 μ M pNP and the indicated additives in the presence of a NADPH-generating system for 10 minutes at 37°C. Inhibition of microsomal cytochrome P450 was achieved by bubbling carbon monoxide through the incubation mixture for approximately 2 minutes prior to the addition of the NADPH-generating system.

^b Numbers in parentheses refer to the per cent inhibition in the presence of the inhibitor(s) as compared to the uninhibited control.

Consistent with a report by Koop (1986), hydroxylation of pNP was shown to be completely independent of the formation of microsomally-formed hydroxyl radicals; in the absence of cytochrome P450 substrates and inhibitors, no effect of desferrioxamine was seen. Carbon monoxide was found to inhibit the reaction by about 60% in the absence and 40% in the presence of desferrioxamine. Substantial inhibition was seen in the presence of both ethanol and DMSO; ethanol inhibiting pNP hydroxylase activity by 74 and 70%, and DMSO by 89% and 85%, in absence and presence of desferrioxamine, respectively. It is noteworthy that the concentration of DMSO employed (i.e. 2 % (v/v)) is a concentration commonly employed to solubilize hydrophobic compounds such as DNP in an aqueous buffer system. This is of

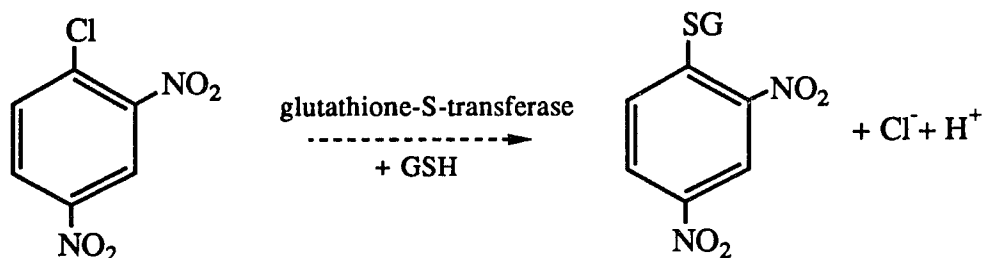
importance as inhibition of CYP2E1 activity by DMSO may affect the metabolism of xenobiotics. Consequently, studies designed to invoke a role of this cytochrome P450 isoform in the metabolism of a xenobiotic should either avoid the use of these solvents or alternatively, the results obtained should be critically evaluated.

2.3.3. Effect of chronic ethanol consumption on cytosolic glutathione-S-transferase activity

GST activities were determined in cytosol from ethanol- and pair-fed animals using 4-nitroquinoline N-oxide as substrate. 1-chloro-2,4-dinitrobenzene (CDNB) was included as a positive control since this GST-substrate shows virtually no discrimination between the various GST isoenzymes (Habig and Jakoby, 1981). The proposed structures of substrates and products are shown in Figure 2.3.

GST activity towards CDNB was found to be increased 1.7-fold ($p < 0.05$) in cytosol from ethanol-fed rats as compared to pair-fed control animals (Table 2.4). GST-catalyzed denitration of 4-NQO was slightly increased ($p < 0.1$) upon ethanol consumption.

A. GSH-conjugation with 1-chloro-2,4-dinitrobenzene:



B. GSH-conjugation with 4-nitroquinoline-N-oxide:

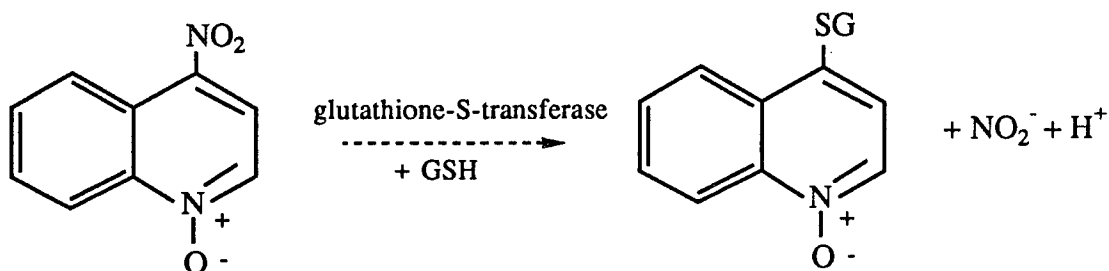


Figure 2.3. GST-catalyzed conjugation of 1-chloro-2,4-dinitrobenzene and 4-nitroquinoline-N-oxide with glutathione. The structures of the GST substrates (A) CDNB and (B) 4-NQO and that of their proposed products after conjugation with glutathione (GSH) are indicated.

Table 2.4. GST activity towards 4-NQO and CDNB in cytosol from ethanol- and pair-fed rats

treatment:	CDNB	4-NQO
	(nmol conjugate/min/mg protein)	(nmol nitrite/min/mg protein)
Pair-fed	1150 ± 140 ^a	390 ± 80 ^a
Ethanol-fed	1910 ± 260 [*]	480 ± 40 ^{**}

^a Results are the mean ± SD of duplicate determinations with 6 individual animals in each treatment group.

^{*} Significantly different from pair-fed control ($p < 0.05$).

^{**} Significantly different from pair-fed control ($p < 0.1$).

2.3.4. Acetylation and deacetylation of the mutagenic amine 2-aminofluorene and isoniazid: Effect of chronic ethanol ingestion¹.

In light of the previously reported results on the effects of ethanol ingestion on cytosolic and microsomal bioactivation of arylamines and DNP (Traynor *et al.*, 1988; Traynor, 1988), it was of interest to determine whether ethanol consumption altered this enzyme activity. Cytosolic N-acetyltransferase (NATase) activity towards two different substrates, isoniazid and the benchmark arylamine 2-aminofluorene (2-AF), and deacetylase activity towards 2-acetylaminofluorene (2-AAF) are shown in Table 2.5. Acetylation of both substrates was unaffected by ethanol consumption, indicating that this activity probably does not account for the different activation profiles by the ethanol

¹ This work has been published as part of a manuscript entitled: Traynor C. A., Shane, B. S., Hajos, A. K. D. and G. W. Winston (1991) Arylamine activation following chronic ethanol ingestion by rats: studies on the liver S9, microsomal and cytosolic fractions and comparison with Aroclor 1254 pretreatment, *Mutat. Res.*, **247**, 153-166. © 1991 Elsevier Science Publishers B. V.

and pair-fed cytosolic systems. Isoniazid-NATase activity was completely inhibited by the presence of 0.1 μM *p*-hydroxymercuribenzoate, which is consistent with the involvement of thiol-groups in the catalytic mechanism of this enzyme (data not shown). Attempts to determine NATase activity towards isoniazid in S-9 fractions were unsuccessful as the reaction was nonlinear and short-lived (less than 1 min) thus, initial velocity could not be accurately assessed. This finding with S9 was probably due to the rapid metabolism of acetyl CoA in these fractions (data not shown) as further additions of acetyl CoA did not alter the kinetic profile of the reaction.

Table 2.5. N-acetyltransferase and deacetylase activities in liver fractions from ethanol- vs. pair-fed rats

	Pair-fed	Ethanol-fed
Cytosolic N-acetyltransferase	(nmol acetylated/min/mg protein)	
2-aminofluorene	0.11 ± 0.05^a	0.11 ± 0.02
isoniazid	1.75 ± 0.20	1.37 ± 0.25
Microsomal deacetylase	(nmol 2-AF from 2-AAF/min/mg protein)	
2-acetylamino- fluorene	1.06 ± 0.11	1.01 ± 0.08

^a Mean \pm S.D. of at least triplicate determinations with cytosol or microsomes from 6 animals of each treatment group. Assays were performed as described in Materials and Methods.

Microsomal deacetylase activity could conceivably account for the pronounced effects of ethanol on the microsomal activation of 2-AAF and 2-aminoanthracene (see Traynor *et al.*, 1991) or the deactivation of DNP (Traynor *et al.*, 1988, Winston *et al.*, 1991), respectively. To test this hypothesis the microsomal deacetylation of one of the substrates, 2-AAF was measured in microsomes obtained from both ethanol and pair-fed rats. The data shown in Table 2.5 indicate that ethanol-ingestion does not alter microsomal deacetylase activity thus, this enzyme probably is not rate limiting.

2.4. Discussion:

Toxic and metabolic effects of ethanol are diverse and complex (see section 1.5). Therefore, to develop an animal model in which metabolic and pathological changes associated with ethanol consumption closely resemble the changes seen in humans, it is necessary to carefully define and control the feeding regimen of the experimental animals. Lieber and DiCarli (1969) developed, and have since improved (Lieber and DiCarli, 1982; 1986), a liquid diet model for rats which greatly fulfills these requirements. Based on their model, the experimental animals used in this study were isocalorically pair-fed a liquid diet containing 36% of the total calories as ethanol for a duration of 30 days. Pair-fed controls received the same daily caloric intake, but with carbohydrate substituted for ethanol.

Rats fed an ethanol-containing diet showed an increase in cytochrome P450 specific content of approximately 50% over isocalorically pair-fed control animals. These values are consistent with values previously reported in the literature (Ryan *et al.*, 1986; Winston and Narayan, 1988).

The increases in microsomal cytochrome b₅ content upon chronic ethanol ingestion coincided with slightly increased levels of microsomal NADH-ferricyanide reductase activity. Ethanol ingestion leads to a dramatic increase in the availability of NADH due to oxidative metabolism of ethanol (Kalant and Khanna, 1969) and the effects of induction of NADH-dependent microsomal reductase activities may be potentiated by the increased availability of reducing equivalents. Rat liver microsomes have been shown to catalyze NADH- and NADPH-dependent reduction of DNP (Heflich *et al.*, 1986; Djuric *et al.*, 1986; 1988). Whereas microsomal NADPH-dependent reduction of DNP is thought to be mediated by cytochrome P450 and cytochrome P450 reductase (Djuric *et*

al., 1988; Shimada and Guengrich, 1990), the NADH-dependent pathway is, at present, unclear. A role for cytochrome b₅ in the NADH-dependent reduction of DNP is one possibility that cannot presently be excluded.

The increase in cytochrome P450 specific content upon ethanol ingestion is likely to be reflective of the induction of ethanol-inducible cytochrome P450 (CYP2E1). Hydroxylation of the nitroarene pNP was chosen to characterize the induction of CYP2E1 in microsomes from ethanol-fed rats. Microsomal fractions from rats pretreated with pyrazole, another potent inducer of CYP2E1 were included as a positive control. Microsomes from ethanol-fed or pyrazole-pretreated rats showed almost 5- and 3-fold increases in the rate of pNP hydroxylation as compared to pair-fed and saline controls, respectively. Ethanol ingestion and pyrazole-pretreatment therefore potently induced CYP2E1 as judged by the greatly increased pNP hydroxylase activity.

Unlike DNP, pNP is water-soluble. The choice of pNP as a substrate for CYP2E1 therefore enabled the investigation of solvent effects on microsomal CYP2E1-dependent activities. This is of importance since many low molecular weight organic solvents (i.e., DMSO, ethanol) are known to be substrates for this P450 isoform (see section 1.5). Further, the same solvents are employed to solubilize DNP in aqueous buffer systems (Djuric *et al.*, 1985; 1986; 1988; Traynor *et al.*, 1988; Shimada and Guengrich, 1990). At solvent concentrations typically employed in these studies, DMSO and ethanol were found to inhibit pNP hydroxylase activity to 89% and 74%, respectively. This potent inhibition of CYP2E1 should be kept in mind when studying the effects of CYP2E1 on the metabolism and mutagenicity of DNP or other toxicants which require the presence of an organic cosolvent.

Microsomes in the presence of divalent metals have been shown to catalyze the NADH- and NADPH-dependent formation of species with the oxidizing power of the

hydroxyl radical (Winston and Cederbaum, 1982; Klein *et al.*, 1983; Kukielka and Cederbaum, 1989; Dicker and Cederbaum, 1990). Rates of formation of hydroxyl radical-like species were shown to be increased with microsomes from ethanol-fed rats (Klein *et al.*, 1983). In turn, these powerful oxidants themselves could affect the ring-hydroxylation of nitroaromatics. pNP hydroxylase activity was therefore determined in the absence and presence of the metal chelator desferrioxamine. Consistent with a previous report (Koon, 1986), hydroxylation of pNP was found to be completely insensitive to the presence of the chelating agent. A role for hydroxyl radical-like species in the microsome-catalyzed ring hydroxylation of pNP could therefore be excluded. In summary, the data presented show that ethanol-ingestion leads to an increase in cytochrome P450 specific content and concomitant induction of CYP2E1. Induction of this isoform is reflected by increased rates of pNP ring-hydroxylation.

Ethanol-consumption not only induces microsomal CYP2E1 but also causes alterations in the specific activity of certain microsomal and cytosolic phase II enzymes, such as GST (see section 2.1). The effect of ethanol on the induction of cytosolic GST's and the consequent effect on nitroarene metabolism was therefore investigated. The data presented here support the notion that while ethanol consumption causes an increase in the total cytosolic GST activity as judged by the increase in CDNB-conjugation, it appears that certain GST isoenzymes present in rat liver are preferentially induced relative to others. The isoenzyme or isoenzymes responsible for GSH-conjugation with 4-NQO do not appear to be within the group of isoenzymes strongly inducible by ethanol. It is therefore unlikely that ethanol-mediated alterations in the metabolism of nitroarenes, such as DNP, can be ascribed to the preferential induction of GST.

The importance of acetylation in the activation of DNP has been discussed (see section 1.3). We have previously reported that 2-AAF activation by S9 at the same toxicant concentration as 2-AF was markedly greater, independent of the pretreatment of the experimental animal (Traynor *et al.*, 1991). A major role for deacetylase activity in the conversion of 2-hydroxy-AAF to its ultimate carcinogenic form has been reported (Lai *et al.*, 1988). The data presented in Table 2.5 indicate that chronic ethanol ingestion does not alter the cytosol-mediated acetyl CoA-dependent acetylation of 2-AF and isoniazid or the microsomal-mediated deacetylation of 2-AAF. Budget analysis of S9 protein has shown that this fraction consists of approximately 20% microsomal and 80% cytosolic protein (Traynor *et al.*, 1991). As shown in Table 2.5, microsomal deacetylase activity towards 2-AAF is almost one order of magnitude higher than is cytosolic NATase activity towards 2-AF at saturating conditions of cofactor and substrate. Taking the differing concentrations of microsomal and cytosolic protein within the S9 fraction into account, deacetylase activity is likely to outweigh NATase activity by approximately two-fold. In conclusion, despite the fact that the previously reported findings clearly indicate that chronic ethanol feeding markedly alters activation of DNP (Traynor *et al.*, 1988; Winston *et al.*, 1991) and arylamines (Traynor *et al.*, 1988; Traynor *et al.*, 1991), it does not appear to be due to differences in acetylation/deacetylation activity by the cytosolic and/or microsomal fractions, respectively.

With respect to the acetylation of hydroxylamino- or aminonitropyrenes, a role of acetylase induction due to ethanol consumption cannot be rigorously excluded. However, Hein and coworkers have very recently reported on acetylator-phenotype dependent rates of arylamine acetylation in rabbit liver (Hein *et al.*, 1991, personal communication). The existence of NATase isoenzymes was indicated by the fact that 2-

AF acetylation was phenotype dependent while that of 1-aminopyrene was not. Acetylation of aminonitropyrenes has not been investigated. It is hence realized that implications on NATase isoenzyme specificity towards aminonitropyrene are merely speculative.

Chapter 3

Dinitropyrene nitroreductase activity of purified NAD(P)H-quinone oxidoreductase: Role in rat liver cytosol and induction by Aroclor-1254 pretreatment[†]

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3.1 Abstract

Dinitropyrenes (DNP*) are potent bacterial mutagens in the Ames test and genotoxins in cultured mammalian cells. Rat liver cytosol contains nitroreductases that are critical in the activation of DNP to the ultimate DNA-binding species. In order to study the nature and inducibility of liver cytosolic enzymes involved in the activation of DNP, cytosolic nitroreductase activities toward three DNP isomers (1,3-, 1,6- and 1,8-DNP) were determined in Aroclor-pretreated and untreated rats. Aroclor-1254-pretreatment resulted in up to 5-fold induction of cytosolic DNP-nitroreductase. This induction was reflected in at least a 15-fold increase in cytosolic NAD(P)H-quinone oxidoreductase (NQOR) [E.C.1.6.99.2] activity. The rates of nitroreduction for the three DNP isomers followed the order 1,6- > 1,8- > 1,3-DNP in all cases studied. 1,6-DNP-nitroreductase co-eluted with NQOR activity upon affinity purification. Highly purified NQOR catalyzed the NADH- and NADPH-dependent reduction of each of the three DNP isomers and displayed the same stereospecificity as did the cytosolic activity. These results provide evidence that NQOR participates in the cytosolic nitroreduction of DNP and constitutes a major part of the total DNP nitroreductase activity upon induction of NQOR by Aroclor-1254-pretreatment. Thus, the role of NQOR in the metabolism of these mutagens depends significantly upon the degree to which this enzyme is induced.

* **Abbreviations:** DNP, dinitropyrene(s); PAHs, polycyclic aromatic hydrocarbons; 1-NP, 1-nitropyrene; NQOR, NAD(P)H-quinone oxidoreductase [E.C.1.6.99.2]; 3-MC, 3-methylcholanthrene; 4-NQO, 4-nitroquinoline-N-oxide; DMSO, dimethylsulfoxide; pNSP, *p*-nitrosophenol; CBA, cibacron blue agarose, SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; DCPIP, 2,6-dichlorophenol-indophenol; sCyt c, partially succinoylated ferricytochrome c.

3.2 Introduction

Dinitropyrenes (DNP) are ubiquitous contaminants in urban air (Nagakawa *et al.*, 1983; Schuetzle, 1983; Tokiwa *et al.*, 1987). DNP are mutagenic, carcinogenic and genotoxic in various animal models (Wilcox and Parry, 1981; Cole *et al.*, 1982; Danford *et al.*, 1982; Rosenkranz and Mermelstein, 1983; 1985, Tokiwa *et al.*, 1984; Fifer *et al.*, 1986; Imaida *et al.*, 1986). They are present in the environment as complex mixtures of other nitropolycyclic aromatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (Nagakawa *et al.*, 1983; Schuetzle, 1983; Tokiwa *et al.*, 1987). These mixtures are known inducers of cytochrome P-450 isoenzymes and various phase II enzyme systems. Therefore, the identity and inducibility of cytosolic and microsomal enzymes involved in the bioactivation of these potent mutagens is of utmost importance. Because they are among the most potent bacterial mutagens known, the mutagenicity of DNP has been extensively studied in the Ames *Salmonella typhimurium* mutagenicity assay (Tokiwa *et al.*, 1981; 1984; 1987; Wilcox and Parry, 1981; Rosenkranz *et al.*, 1982; Schuetzle, 1983; Shah *et al.*, 1990). As they do not require exogenous enzyme sources for mutagenic expression in *S. typhimurium*, DNP are sometimes termed direct-acting mutagens. However, nitroreduction is required for activation to the ultimate mutagen as evinced by the reduced mutagenic response of 1-nitropyrene and 1,3-DNP in strain TA98NR which lacks the so-called classical nitroreductase (Rosenkranz *et al.*, 1982; Eddy *et al.*, 1986). In contrast, 1,6- and 1,8-DNP are thought to be reduced by other nitroreductases and retain almost full mutagenic potential in *S. typhimurium* TA98NR (Klopman *et al.*, 1984). This substrate selectivity is thought to be due to the preferential one-electron reduction of 1-NP and 1,3-DNP by the classical nitroreductase, based upon the electrochemical reduction preferences of these two compounds (Eddy *et al.*, 1986). It has subsequently been shown that

TA98NR displays lowered nitroreductase activity towards 1-NP and 1,3-DNP, but not 1,6- or 1,8-DNP (Djuric *et al.*, 1986).

The Ames mutagenicity assay has also been employed to study the metabolism of DNP by rat hepatic S9 as well as microsomal and cytosolic fractions (Tokiwa *et al.*, 1981; Traynor *et al.*, 1988; Shah *et al.*, 1990). Cytosolic and microsomal reductive metabolites have been identified in liver and lung for 1,3-, 1,6- and 1,8-DNP (Rosenkranz and Mermelstein, 1985; Djuric *et al.*, 1986; Heflich *et al.*, 1986; Djuric *et al.*, 1988; Tee *et al.*, 1988). The major DNA adduct identified from 1,8-DNP in bacterial and mammalian systems is N-(deoxyguanosin-8-yl)-1-amino-8-nitropyrene *in vivo* (Andrews *et al.*, 1986; Djuric *et al.*, 1988; Norman *et al.*, 1989) and *in vitro* (Andrews *et al.*, 1986). The initial nitroreduction is believed to be the rate-limiting step in the activation of DNP and similar nitrated polycyclic aromatic hydrocarbons in mammalian systems (Fifer *et al.*, 1986; Tee *et al.*, 1988; Norman *et al.*, 1989). In turn, esterification has been suggested to be the critical step in the bacterial activation of DNP (Fifer *et al.*, 1986).

The cytosolic enzymes aldehyde oxidase, xanthine oxidase and NAD(P)H-quinone oxidoreductase (NQOR) have all been suggested to be involved in the reduction of DNP to the proximate mutagen (Rosenkranz and Mermelstein, 1983; Djuric *et al.*, 1985; 1988; Belisario *et al.*, 1990; Djuric and McGunagle, 1989). NQOR has been referred to as a phase II enzyme because of its role in the detoxification of redox-cycling quinones and PAH metabolites after phase I metabolism (Lind *et al.*, 1978; 1982). In contrast to other flavin-linked reductases such as microsomal NADPH-P450 oxidoreductase or cytosolic xanthine oxidase, NQOR catalyzes two-electron reduction of its substrates and hence leads to the formation of more oxygen-stable metabolites. Purified NQOR catalyzes the reduction of the prooxidant 4-nitroquinoline N-oxide (4-

NQO) (DeFlora *et al.*, 1988), certain azodyes (Huang *et al.*, 1979) and aromatic C-nitrosocompounds (Horie *et al.*, 1982). Nitroreductase activity associated with bacterial (Tatsumi *et al.*, 1981) and rat liver cytosolic NQOR (Fukuda *et al.*, 1972) is suggested by coelution of quinone- and nitroreductase activities upon partial purification. Purified NQOR has been shown to decrease the mutagenicity of 4-NQO, among a variety of other compounds (DeFlora *et al.*, 1988). Rat liver cytosolic NQOR is co-induced with arylhydrocarbon hydroxylase and glutathione-S-transferases by pretreatment with inducers causing the activation of the Ah-locus e.g. 3-methylcholanthrene or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Robertson *et al.*, 1986). The precise mechanism of induction of NQOR has not been clearly established (Robertson *et al.*, 1986; Prochaska and Talalay, 1988) however, the existence of multiple genes in rat and human liver suggests the presence of multiple forms of NQOR (Robertson *et al.*, 1986; Jaiswal *et al.*, 1990). Two isofunctional forms have been purified from mouse liver (Prochaska and Talalay, 1986).

Herein, the role of NQOR in the reductive metabolism of DNP by rat liver cytosol was investigated. NQOR was found to possess DNP-nitroreductase activity and to exert stereoselectivity towards the three DNP isomers studied. The results suggest that the contribution of this enzyme to the total nitroreductase activity of rat liver cytosol depends significantly upon its degree of induction.

3.3. Materials and Methods

3.3.1. Materials

Allopurinol, bovine serum albumin, cibacron blue 3GA-agarose, 2,6-dichlorophenol-indophenol, dicoumarol, ferricytochrome c, hypoxanthine, NADH,

NADPH, Tris-base, Tris-Cl, and xanthine oxidase were from Sigma (St. Louis, MO). 1,3-, 1,6- and 1,8-dinitropyrene were from Chemsyn Science Laboratories (Lenexa, KS) (>99% purity). Menadione bisulfite, *p*-nitrosophenol and succinic anhydride were from Aldrich Chemical Company (Milwaukee, WI). Aroclor-1254 was from Foxboro Analabs (North Haven, CT). Partially succinoylated ferricytochrome was prepared according to the method of Kuthan *et al.* (1982). In three different preparations, succinoylation was determined to be 44-52% complete as shown by the decrease in trinitrobenzenesulfonate reactive amino groups. This degree of succinoylation virtually destroys its suitability as a substrate for microsomal NAD(P)H-dependent cytochrome c reductase activities (Kuthan *et al.*, 1982) as evinced by the fact that the reduction of partially succinoylated cytochrome c by NADPH-supplemented microsomes is almost completely inhibitable by superoxide dismutase.

3.3.2. *Animals and Treatment*

Male Sprague-Dawley rats were obtained from Hilltop Animals Inc., (Scottsdale, PA). Rats were maintained in stainless steel cages on a 12-hour light/dark cycle in a temperature and humidity-controlled environment and fed a diet of Purina Rat Chow *ad libitum* until they reached a weight of 150-200 g. Aroclor 1254 was given intraperitoneally (IP) in corn oil as a single injection at 500 mg/kg body weight five days prior to sacrifice. 3-Methylcholanthrene and pyrazole were given IP (at 35 mg/kg in corn oil and saline, respectively) on day 1, 3 and 5 prior to sacrifice. Rats were killed by decapitation, livers were removed and liver fractions prepared by differential centrifugation as described previously (Winston and Narayan, 1988). The 105,000 x g supernatant was taken as cytosol. All tissue fractions were stored at -70°C prior to use.

3.3.3. Enzyme assays

DNP were dissolved at approximately 0.3 mg/ml in dimethylsulfoxide (DMSO) and stored under Argon at -70°C. Concentrations of stock solutions were determined spectrophotometrically using the extinction coefficients provided by the supplier. Aerobic cytosolic nitroreductase activities were measured essentially as outlined by Djuric *et al.* (1986). The incubation mixture contained 20 µM succinoylated ferricytochrome c, 0.05-0.11 mg cytosolic protein, 1 mM cofactor and 20 µM dinitropyrene dissolved in 50 mM potassium phosphate buffer, pH 7.4 to a final volume of 1 ml. The reaction was initiated by the addition of either substrate or enzyme. Some experiments additionally contained dicoumarol (100 µM) or allopurinol (10 µM). In pilot experiments, these inhibitor concentrations were found to inhibit NQOR- (menadione as substrate) and xanthine oxidase (xanthine as substrate) activity by greater than 95%. Reduction of the succinoylated cytochrome c was monitored spectrophotometrically at 37°C by following the increase in absorbance at 550 nm against a reference, which contained all reactants but the dinitropyrene. An extinction coefficient of $E_{550\text{ nm}} = 21\text{ mM}^{-1}\text{ cm}^{-1}$ was used for quantification (VanGelder and Slater, 1962). NAD(P)H-quinone oxidoreductase activity was determined as described by Ernster (1969) using 40 µM dichlorophenol-indophenol or 20 µM menadione as the substrate and 50 µM ferricytochrome c (Cyt. c) as the terminal electron acceptor in the presence of 200 µM NADH or NADPH. The buffer was 50 mM Tris-Cl, pH 7.4 containing 0.2 % (v/v) Tween 20 and 0.5 mM KCN. The inhibition of the xanthine oxidase-catalyzed conversion of xanthine to urate by allopurinol and dicoumarol was determined by following the change in absorbance at 295 nm as described by

Rajagopalan (1985). C-nitrosoreductase activity was determined by following the oxidation of NADH spectrophotometrically at 340 nm in the presence of p-nitrosophenol (pNSP) as substrate in the same buffer used for NQOR activity assays. The extinction coefficient was corrected for the loss in absorbance at 340 nm due to disappearance of pNSP (Horie *et al.*, 1982). Protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as the reference standard.

3.3.4. Purification of NAD(P)H-quinone oxidoreductase

Rat liver cytosolic NQOR was purified from Aroclor- or pyrazole-pretreated rats by the method of Prochaska (1988). There was absolutely no difference between the NQOR purified from Aroclor- or pyrazole-treated rats in either activity or electrophoretic mobility. Cytosol from 6 rats was incubated with 20 ml preconditioned cibacron blue agarose (CBA) at room temperature on an orbital shaker for 90 minutes. Small aliquots were removed from the solution in order to assay disappearance of enzyme activity from the supernatant. These were centrifuged and separated immediately to prevent further binding to CBA. After 90 minutes, the gel was allowed to settle, the supernatant decanted and the gel transferred to a column (20 x 1.75 cm). All subsequent steps were performed at 4°C. After extensive washing with 50 mM Tris-Cl, 3.5 M NaCl, pH 7.4 (buffer A), 5 mM Tris-Cl, pH 7.4 (buffer B) and subsequently 30 mM Tris-Cl, pH 10 containing 50 % (v/v) ethylene glycol (buffer C), the column was eluted with 1 mM NADH in 20 mM Tris, 50% ethylene glycol, pH 10. Fractions (5 ml) were assayed for protein concentration and reductase activities as indicated. Those with peak activities were pooled, washed and concentrated on an Amicon PM-10 ultrafiltration membrane

and subsequently in an Amicon B-15 ultrafiltration chamber. The bright yellow concentrate was stored frozen in the presence of 30% (v/v) glycerol.

3.3.5. SDS-PAGE of cibacron blue-binding cytosolic proteins

To test for induction of total NQOR protein in rat liver cytosol by SDS-PAGE, a small scale partial purification procedure was designed as follows: 50 mg of rat liver cytosolic protein from Chow-fed, 3-methylcholanthrene (3-MC)- and Aroclor-pretreated animals was incubated with preconditioned CBA (5 mg dry weight) at 4°C for 1 hour while shaking. This was centrifuged and the supernatant decanted. The gel was washed with 5 ml each of 0.15 M NaCl, buffer A and C. Subsequently, CBA-bound proteins were eluted with twice 1.5 ml 1 mM NADH in buffer C. The combined fractions were concentrated by means of ultrafiltration, lyophilized to dryness and redissolved to a final volume of 40 µl. The equivalent of 14% of the total sample (corresponding to 6.8 mg cytosolic protein) of each preparation was loaded onto a 12% SDS-polyacrylamide gel. SDS-PAGE was carried out according to Laemmli (1970) and gels were stained with Coomassie Blue.

3.4. Results

3.4.1. Induction with Aroclor 1254

Pretreatment of rats with Aroclor 1254, a potent mixed inducer of the microsomal mixed function oxygenase system, was found to increase cytosolic NQOR activity over noninduced controls by approximately 25- and 15-fold with respectively

DCPIP and menadione as substrates (Table 3.1). No statistically significant differences were observed in the NADH- vs. NADPH-dependent activities with either substrate or pretreatment. Induction of cytosolic NQOR by Aroclor-pretreatment has previously been reported using ethoxyresorufin as substrate (Nims *et al.*, 1984). SDS-PAGE shows that the increase in NQOR activity in cytosol from Aroclor-pretreated animals is associated with an increase in a 36 kDa band (Figure 3.1). NQOR was partially purified using a revised small scale purification procedure as described in Materials and Methods. Cytosol from 3-MC-pretreated rats was included as a positive control. An increase in the intensity of staining of a polypeptide band corresponding to a molecular weight of approximately 36 kDa was seen in cytosol from 3-MC- (lane 2) and Aroclor-pretreated animals (lane 3) compared to chow-fed controls (lane 1). The bands comigrated with purified NQOR (lane 4). It is noteworthy, that there are three other bands, two corresponding to approximately 25 kDa and one corresponding to about 50 kDa, which appear to be increased upon pretreatment of the animal with Aroclor-1254.

Table 3.1. NAD(P)H-quinone oxidoreductase activity in rat liver cytosol

		$\mu\text{moles DCPIP reduced}$ $\text{min} \times \text{mg protein}$	$\mu\text{moles Cyt. c reduced}$ $\text{min} \times \text{mg protein}$
Chow-fed	NADH	0.19 ± 0.01^a	0.37 ± 0.05
	NADPH	0.22 ± 0.01	0.36 ± 0.01
Aroclor	NADH	5.15 ± 0.43	4.75 ± 0.29
	NADPH	4.76 ± 0.26	5.52 ± 0.32

^a Results are the mean \pm SD of at least triplicate determinations with pooled cytosol from 3-4 rats. Rates represent the dicoumarol-uninhibited minus -inhibited rate. Experimental details are as indicated in Materials and methods.

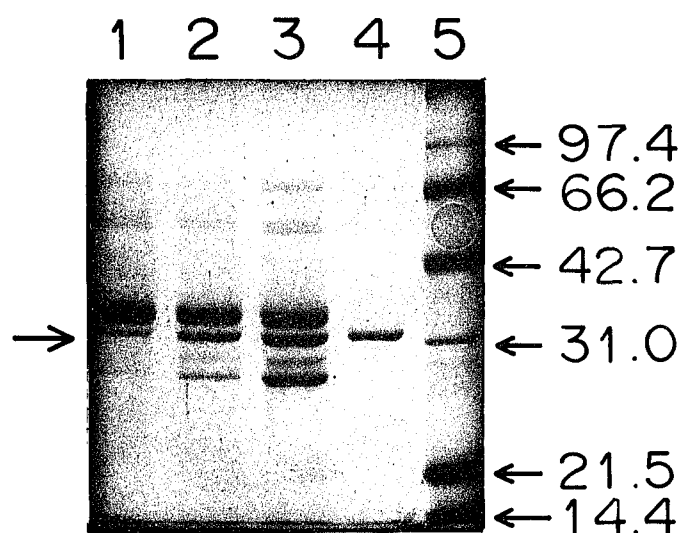


Fig. 3.1: SDS-polyacrylamide gel electrophoresis of rat liver cytosol after partial purification of NQOR from chow-fed, 3-MC- and Aroclor-pretreated rats. Samples were prepared as described in Materials and methods. Lane 1. chow-fed control Lane 2. 3-MC-induced Lane 3. Aroclor-1254-pretreated Lane 4. purified NQOR (approximately 1.5 μ g protein) Lane 5. molecular weight markers. Numbers on the right refer to molecular mass (in kDa) standards. The position of the 36 kDa purified NQOR is indicated by the arrow on the left.

3.4.2. Cytosolic DNP nitroreductase

DNP-Mediated reduction of partially succinoylated cytochrome c (sCyt c) was shown to be catalyzed by cytosolic preparations from both control and induced rats (Table 3.2). While nitroreduction also occurred in the absence of added cofactor, this rate was less than 25% of the rate in the presence of either NADH or NADPH (data not shown). The results obtained with cytosol from chow-fed controls agree reasonably well with those reported for 1,3- and 1,6-DNP by Djuric *et al.* (1986). In all cases the rate of reduction of 1,6-DNP was greatest followed by 1,8- and 1,3-DNP.

Table 3.2. Nitroreduction of DNP by rat liver cytosol

Treatment	cofactor	1,3-DNP	1,6-DNP	1,8-DNP
		(nmol sCyt c reduced/min/mg protein)		
Chow-fed	NADPH	0.8 ± 0.1 ^a	7.0 ± 0.4	6.0 ± 0.3
	NADH	0.8 ± 0.1	7.1 ± 0.7	6.2 ± 0.5
Aroclor	NADPH	3.5 ± 0.5 (4.7) ^b	7.8 ± 1.4 (1.1)	6.7 ± 0.6 (1.1)
	NADH	2.5 ± 0.1 (3.2)	22.0 ± 0.3 (3.1)	13.0 ± 0.6 (2.1)

^a Results are mean ± SD of at least 3-5 determinations with cytosol pooled from 3 Aroclor-pretreated or 4 control rats.

^b Numbers in parentheses refer to the fold-induction of nitroreductase activity over chow-fed controls.

NADPH-dependent activities: Table 3.2 shows that 1,3-DNP-nitroreductase activity in cytosol from Aroclor-pretreated animals was increased 4.7-fold compared to chow-fed controls. No induction of nitroreductase activity toward 1,6-DNP or 1,8-DNP was

observed. To further elucidate the nature of this apparent stereoselectivity of DNP-reductase, incubations were performed in the presence of dicoumarol, a potent inhibitor of NQOR and allopurinol, a suicide inhibitor of the xanthine oxidase / xanthine dehydrogenase couple (Table 3.3). In control experiments with purified xanthine oxidase it was found that 100 μ M dicoumarol inhibited the xanthine oxidase-catalyzed conversion of xanthine to urate by 50%. As 50% inhibition by 100 μ M dicoumarol was the maximum level of inhibition of xanthine oxidase the per cent inhibition shown in Table 3.3 was corrected by subtracting 50% of the allopurinol-sensitive rate from the portion of the rate inhibitable by 100 μ M dicoumarol. As judged from the differential rates in the presence or absence of these inhibitors, NADPH-dependent reduction of 1,6- and 1,8-DNP is about equally dependent on xanthine oxidase and NQOR in both cytosolic systems (Table 3.3). In contrast, 72% of the reduction of 1,3-DNP catalyzed by cytosol from Aroclor-pretreated animals was inhibitable by dicoumarol, while only 26% inhibition was seen in the control system. Thus, the increase in 1,3-DNP nitroreductase activity by Aroclor-pretreatment can be ascribed to increased cytosolic NQOR activity. Allopurinol was a potent inhibitor of the reduction of 1,6- and 1,8-dinitropyrene catalyzed by both cytosolic systems however, it was without effect on the reduction of 1,3-DNP by the control cytosol and only slightly inhibited reduction by cytosol from Aroclor-pretreated animals.

Table 3.3 Nitroreduction of DNP by rat liver cytosol: Effect of inhibitors

NADPH-dependent activity:

		1,3-DNP	1,6-DNP	1,8-DNP
Treatment inhibitor		(nmol sCyt c reduced/min/mg protein)		
Chow	dicoumarol	0.6 ± 0.3 ^a (26%) ^b	2.7 ± 0.2 (44%)	2.1 ± 0.5 (41%)
	allopurinol	0.9 ± 0.1 (0%)	4.4 ± 0.2 (37%)	3.2 ± 0.2 (47%)
Aroclor	dicoumarol	0.6 ± 0.2 (72%)	2.4 ± 0.3 (45%)	1.1 ± 0.3 (58%)
	allopurinol	2.6 ± 0.3 (25%)	4.1 ± 0.5 (48%)	3.2 ± 0.2 (53%)

NADH-dependent activity:

		1,3-DNP	1,6-DNP	1,8-DNP
Treatment inhibitor		(nmol sCyt c reduced/min/mg protein)		
Chow	dicoumarol	0.4 ± 0.1 ^a (33%) ^b	2.0 ± 0.4 (41%)	1.9 ± 0.4 (51%)
	allopurinol	0.6 ± 0.1 (20%)	2.7 ± 0.1 (62%)	3.9 ± 0.6 (37%)
Aroclor	dicoumarol	0.6 ± 0.2 (54%)	2.4 ± 0.3 (45%)	2.5 ± 0.1 (55%)
	allopurinol	1.4 ± 0.2 (43%)	4.1 ± 0.5 (48%)	6.4 ± 0.3 (51%)

^a The results are expressed as the mean ± SD of at least triplicate determinations.^b Corrected per cent inhibition of the rates of nitroreduction as compared to the absence of 100 µM dicoumarol or 10 µM allopurinol, respectively. The dicoumarol-sensitive part of the reaction was corrected for the observed partial inhibition of xanthine oxidase by dicoumarol. For further details, see Results.

NADH-dependent activities: The NADH-dependent reduction of all three DNP isomers was induced 2-3-fold by pretreatment with Aroclor-1254. Depending on the isomer used, dicoumarol inhibited the reduction of DNP by cytosol from control rats 33-51% and that by Aroclor-pretreated rats 45-55% (Table 3.3, Figure 3.2). The dicoumarol-sensitive rates for 1,3-, 1,6- and 1,8-DNP were hence, 1.4, 12.3 and 7.2 versus 0.3, 2.9 and 3.2 nmol sCyt c reduced/min/mg protein, respectively in the Aroclor as compared to the chow-fed cytosolic system. Rates of DNP reduction in the presence of dicoumarol were found to be virtually identical in both cytosolic preparations which supports the assertion that increased reductive metabolism of DNP is due at least in part to induction of cytosolic NQOR.

3.4.3. *Studies on purified NQOR*

To further elucidate the role of NQOR in DNP-reduction by cytosol, cytosolic NQOR was purified by biospecific absorption on cibacron blue agarose. More than 90% of the NADH-dependent menadione oxidoreductase and 95% of the NADH-dependent *p*-nitrosophenol (pNSP) C-nitrosoreductase activity bound to the affinity gel within the first 15 minutes. This characteristic binding of NQOR has been reported previously (Prochaska, 1988). The loss of quinone reductase- and C-nitrosoreductase-activity was reflected in a loss of 60% of the NADH-dependent 1,6-DNP nitroreductase activity in the Aroclor cytosolic supernatant. This remaining nitroreductase activity was insensitive to dicoumarol (not shown) and was thus not due to NQOR but to other cytosolic nitroreductase activities which do not bind to CBA. After extensive washing of the gel, the flavoenzyme was eluted with 100 ml of 1 mM NADH. A single protein peak associated with NQOR (NADH-menadione oxidoreductase) activity was eluted (Figure

3.2a). This activity coeluted with pNSP C-nitrosoreductase activity (Figure 3.2b) and 1,6-DNP nitroreductase activity (Figure 3.2c). The purified enzyme showed the characteristic absorption spectrum of a flavoenzyme (not shown) (Huang *et al.*, 1979). The preparation was pure by criterion of homogeneity on SDS-PAGE (Figure 3.1) and the monomeric molecular weight was estimated at 36 kD, consistent with previous reports (Robertson *et al.*, 1986, Prochaska, 1988).

Purified NQOR catalyzed the nitroreduction of all three DNP isomers (Figure 3.3). The NADH-dependent rate of reduction followed the order 1,6- > 1,8- > 1,3-DNP and the order of the NADPH-dependent rate was 1,6- > 1,3- ≥ 1,8-DNP. The rate of nitroreduction increased linearly up to at least 40 μM with all three DNP (data not shown).

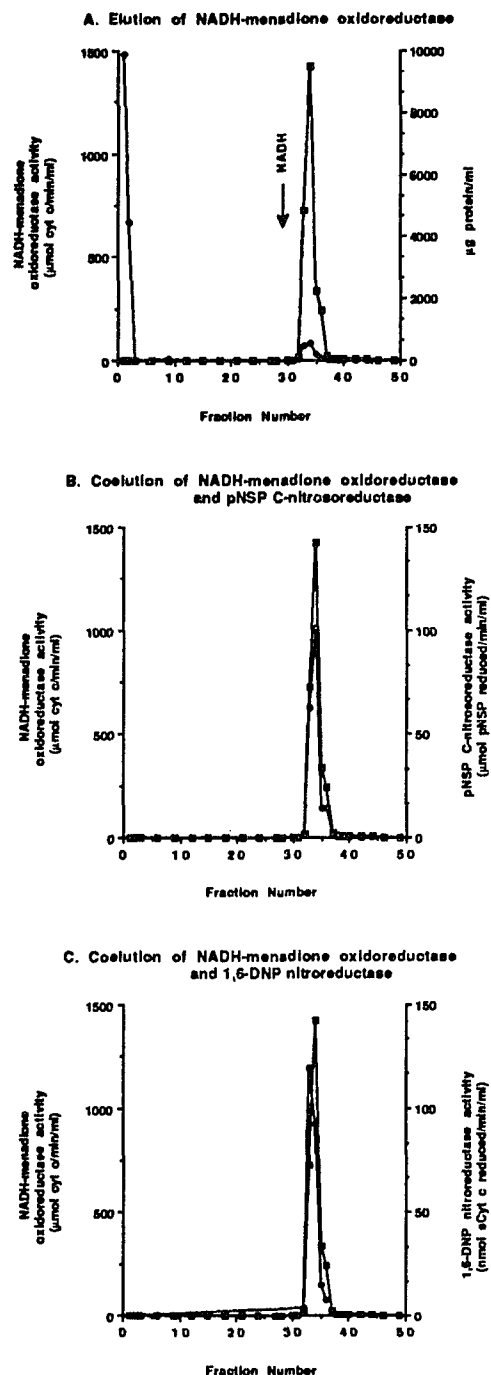


Fig. 3.2: Purification of NQOR from cytosol from Aroclor-pretreated rats. A) NADH-menadione oxidoreductase (open squares) elutes as a single protein peak (diamonds). B) Coelution of NADH-menadione oxidoreductase (open squares) and p-nitroso-phenol C-nitrosoreductase activity (open circles). C) Coelution of NADH-menadione (open squares) and 1,6-DNP nitroreductase activity (closed circles). Experimental details as well as those for the purification of NQOR are described in Materials and methods.

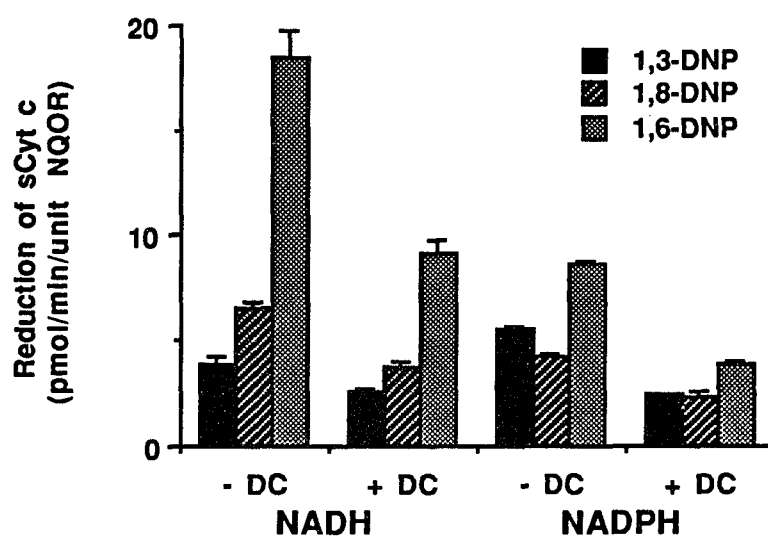


Fig. 3.3: NADH- and NADPH-dependent reduction of 1,6-, 1,8- and 1,3-DNP by purified NQOR and inhibition by dicoumarol. DNP was incubated in the presence of approximately 150 units of NQOR (as determined with DCPIP as the substrate) and 200 μ M NADH in presence or absence of 100 μ M dicoumarol (DC). DNP nitroreduction was measured as the DNP-dependent reduction of sCyt c as described in Materials and methods.

3.5. Discussion

We have investigated the role of cytosolic NQOR in the metabolism of DNP. Djuric *et al.* (1988) reported on the induction of cytosolic and microsomal DNP-nitroreductase activities by pretreatment of rats with 1-nitropyrene (1-NP). Their data suggested that this pretreatment results in about a 2-fold increase in the xanthine oxidase and aldehyde oxidase-mediated reduction of 1-NP. Furthermore, 1-NP-pretreatment increased 1,6-DNP reduction to partially reduced metabolites by approximately 2-fold, but this increase could not clearly be ascribed to the induction of any specific enzyme. While the ability of various cytosolic enzymes to reduce DNP has been implied by the use of selective inhibitors and cofactors (Djuric *et al.*, 1988), the role of these in the activation to the penultimate mutagenic species has not yet been clarified. Xanthine oxidase has been shown to catalyze the reduction of 1-NP and 1,6-DNP to metabolites capable of binding to DNA (Howard and Beland, 1982; Howard *et al.*, 1985; Djuric and McGunagle, 1989). More recently, Djuric and McGunagle reported on the fractionation of a cytosolic factor with 1,6-DNP-nitroreductase activity, although this factor was not identified (Djuric and McGunagle, 1989). The present findings show that NQOR purified from rat liver cytosol exhibits NADH- and NADPH-dependent nitroreductase activity towards 1,3-, 1,6- and 1,8-DNP. Rates of nitroreduction followed the order 1,6-DNP > 1,8-DNP > 1,3-DNP. Both the specific activity and specific protein content of cytosolic NQOR is potently induced upon pretreatment of the animal with Aroclor-1254. This induction is reflected in the increased rates of NAD(P)H-dependent DNP reduction. Differential effects were noted however, depending on the DNP isomer and the cofactor employed. NADH-dependent nitroreductase activity towards all three DNP-isomers was found to be increased 2-3 fold. In contrast, an increase in NADPH-

dependent nitroreduction (5-fold) was seen only towards 1,3-DNP. The observed cofactor and substrate specificities displayed suggest the involvement of other, as yet unidentified nitroreductases, which efficiently compete with cytosolic NQOR and display stereoselectivity or the presence of cytosolic NQOR isoenzymes with catalytic preferences towards specific DNP-isomers and cofactor utilization. The latter probably has greater credence in light of recent reports on the existence of multiple genes for NQOR in humans and the existence of at least two isoenzymes in rat and mouse liver (Prochaska and Talalay, 1986; Jaiswal *et al.*, 1990; and references therein). It is not known however, whether Aroclor 1254 displays selectivity in the induction of NQOR isoenzymes and the existence of other, as yet uncharacterized nitroreductases cannot be rigorously excluded. The present findings also indicate that caution should be exercised when inhibitors are used to assign enzyme activities in crude fractions. Dicoumarol, at concentrations commonly employed to inhibit NQOR activity, is a rather potent inhibitor of purified xanthine oxidase (see Appendix). Such overlapping inhibitor specificities might prevent unequivocal assignment of the involved enzymatic activities.

NQOR catalyzes the committed two-electron reduction of quinones or nitrocompounds thereby preventing the formation of more unstable, univalently reduced metabolites which can undergo autooxidation and redox cycling. In this regard, microsomal NADPH-P450 reductase, a one-electron reducing enzyme, has recently been reported to decrease the direct-acting mutagenicity of 1,6- and 1,8-, but not 1,3-DNP (Shimada and Guengrich, 1990). Another one-electron transferring enzyme, xanthine oxidase, forms oxygen-unstable products from 1,6- and 1,8-DNP. In contrast to 1,6- and 1,8-DNP which are preferentially reduced by two electrons, 1,3-DNP is preferentially reduced by one electron. Thus, cytosolic NQOR might efficiently compete

with microsomal or cytosolic one-electron nitroreductases and thereby prevent the formation of one-electron reduced metabolites.

DNP nitroreductase is shown herein to be an inducible cytosolic activity which is associated with the induction of NQOR and most likely, the only one that catalyzes two-electron transfer to the nitro-group. The present data do not exclude the possibility that other NQOR isoenzymes exist which might contribute to the two-electron nitroreduction of DNP and are inducible upon animal pretreatment with Aroclor 1254. Induction of this reductive pathway deserves consideration as we (Traynor *et al.*, 1988) and others (Shah *et al.*, 1990) have previously reported that rat liver cytosol can augment or negate the mutagenicity of DNP in the Ames test, depending on the pretreatment of the animal and the DNP isomer employed.

While nitroreduction is clearly required for DNA-adduct formation and expression of the mutagenicity of DNP, other pathways such as O-acetylation are also of importance (Djuric *et al.*, 1985; 1988). This activation step needs to be considered in conjunction with, rather than isolated from, reductive pathways as changes in the rates of nitroreduction may result in either an increase of penultimate mutagens (i.e. nitroso- or hydroxylaminonitropyrenes) or in the formation of metabolites of rather low mutagenic potential, such as the more completely reduced aminonitro- or diaminopyrenes.

3.6. Acknowledgements

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Chapter 4

Purified NAD(P)H-quinone oxidoreductase enhances the mutagenicity of dinitropyrenes *in vitro*[†]

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4.1. Abstract:

The effect of highly purified rat liver cytosolic NAD(P)H-quinone oxidoreductase [E.C. 1.6.99.2] on the mutagenicity of 1,3-, 1,6- and 1,8-dinitropyrene (DNP) was studied in the Ames *Salmonella typhimurium* mutagenicity assay. NAD(P)H-quinone oxidoreductase over the range of 0.02 - 0.8 µg/plate (38-1500 units) increased up to 3-fold the mutagenicity of all three DNP in *S. typhimurium* TA 98. In TA98NR, a strain deficient in "classical" nitroreductase, the mutagenicity of 1,6- and 1,8-DNP was essentially unchanged, whereas that of 1,3-DNP was markedly reduced. NAD(P)H-quinone oxidoreductase enhanced the mutagenicity of 1,6- and 1,8-DNP to approximately equivalent extents in TA98NR and TA98. The mutagenicity of 1,3-DNP in TA98NR was potently enhanced by the addition of NAD(P)H-quinone oxidoreductase in a dose-responsive manner. In the presence of 0.8 µg NAD(P)H-quinone oxidoreductase, 1,3-DNP displayed a mutagenic response in TA98NR that was comparable to that obtained in TA98. NAD(P)H-quinone oxidoreductase was found to increase the mutagenicity of 1,6- but not 1,3- or 1,8-DNP to mutagenic intermediates in TA98/1,8-DNP₆, a strain deficient in O-acetyltransferase activity. The results suggest that NAD(P)H-quinone oxidoreductase not only catalyzes reduction of the parent DNP but also that of partially reduced metabolites generated from that DNP. Such reductive metabolism may lead to increased formation of the penultimate mutagenic species.

4.2. Introduction

Dinitropyrenes (DNP) are genotoxic in various animal models and are amongst the most potent bacterial mutagens known (Rosenkranz and Mermelstein, 1983; Tokiwa *et al.*, 1990). Activation of DNP to the ultimate DNA-binding species is thought to be mediated by nitroreduction and at least in some instances by subsequent N- and O-acetylation of the partially reduced hydroxylamino-nitropyrenes (Rosenkranz and Mermelstein, 1983; McCoy *et al.*, 1983; Djuric *et al.*, 1985; Howard *et al.*, 1987). A differential role for one- and two-electron reduction of DNP in the bacterial metabolism of DNP has been suggested (Howard *et al.*, 1987). A mutant strain of *Salmonella typhimurium*, TA98NR, was selected which lacks a one-electron transferring nitroreductase. This strain has specificity toward reduction of 1,6- and 1,8-DNP rather than 1,3-DNP; therefore the mutagenicity of only the 1,3-DNP isomer is strongly suppressed (Rosenkranz and Mermelstein, 1983). Evidence for preferred one-electron reduction of 1,3-DNP comes from its electrochemical reduction characteristics. In contrast, 1,6- and 1,8-DNP are thought to be reduced by an initial two-electron transfer (Howard *et al.*, 1987; Klopman *et al.*, 1984).

NAD(P)H-quinone oxidoreductase (NQOR) is a flavoenzyme capable of the two-electron reduction of a variety of organic and inorganic compounds (DeFlora *et al.*, 1988 and references therein). This enzyme, amongst other cytosolic and microsomal activities, has been suggested to be involved in the nitroreduction of DNP in rat liver cytosol and post-mitochondrial 9000 x g supernatant (Djuric *et al.*, 1986; 1988) and of the monofunctional alkylating agent CB1954 (Knox *et al.*, 1988). NQOR is highly inducible in rats upon pretreatment with 3-methylcholanthrene (Williams *et al.*, 1986 and references therein) or the polychlorinated biphenyl mixture, Aroclor 1254 (Nims *et al.*,

1984; Hajos and Winston, 1990; 1991). We have recently reported that DNP nitroreductase activity co-elutes with NAD(P)H-menadione oxidoreductase activity from a cibacron blue affinity column, is induced in concomitance with induction of NQOR, and is inhibitable by dicoumarol (Hajos and Winston, 1990; 1991). Upon induction, this activity contributes substantially to the total cytosolic DNP nitroreductase activity (Hajos and Winston, 1991). The present study was undertaken to clarify the role of cytosolic NQOR in the mutagenic expression of DNP *in vitro*.

4.3. Materials and Methods

4.3.1. Chemicals and reagents: 1,3-, 1,6-, 1,8-dinitropyrene and 2-nitrofluorene (purity $\geq 98\%$ for all) were from Aldrich Chemical Company (Milwaukee, WI) and were stored at -70°C in dimethylsulfoxide (DMSO). Ferricytochrome *c*, menadione bisulfite, NADP^+ , NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, dicoumarol, DMSO, potassium phosphate, Tris-Cl, MgCl_2 , L-histidine and D-biotin were from Sigma Chemical Co. (St. Louis, MO). Sodium ammonium phosphate and dextrose were obtained from Fisher Scientific (Springfield, NJ); Oxoid Nutrient Broth No. 2 was from KC Biologicals, Inc. (Lenexa, KS) and Noble agar from Difco Laboratories (Detroit, MI). Salmonella strain TA98 was the generous gift of Dr. Bruce N. Ames, University of California, Berkeley, CA and the strains TA98NR and TA98/1,8-DNP₆ were likewise the generous gifts of Dr. E. C. McCoy, Case Western Reserve University, School of Medicine, Cleveland, OH.

4.3.2. Purification of NAD(P)H:quinone oxidoreductase and determination of specific activity: NAD(P)H:quinone oxidoreductase ([E.C.1.6.99.2]) was purified from rat liver cytosol according to the procedure of Prochaska (1988). The preparation was pure as judged by the appearance of only a single polypeptide band of 36 kDa upon SDS polyacrylamide gel electrophoresis. NQOR activity was determined in a final reaction volume of 1 ml. Incubations contained 200 μM NADPH, 40 μM menadione and 50 μM ferricytochrome *c* in 50 mM Tris-HCl pH 7.4 containing 0.2% (v/v) Tween-20 and 0.5 mM KCN (Ernster, 1969). One unit of NQOR is defined here as the amount of enzyme which catalyzes the reduction of 1 nmol ferricytochrome *c* per minute. The preparation used had a specific activity of approximately 1900 kunits/mg protein.

4.3.3. Ames Salmonella mutagenicity assay: The Ames *Salmonella* mutagenicity assay was performed according to the revised plate incorporation procedure of Maron and Ames (1983). Specific amounts of purified NQOR were premixed with a NADPH-generating system in 0.2 M sodium phosphate buffer pH 7.4. The mixture was sterilized by filtration through a 0.2 μ M filter and then mixed with bacteria (approximately 10^8 organisms), toxicant and top agar before finally being poured onto minimal-glucose plates. Following 48 hours incubation at 37°C, these plates were scored for revertants using a Biotran II Colony Counter. 2-Nitrofluorene (5 μ g/plate) was routinely included as a positive control for mutagenesis (typical revertant frequency in TA98 = 850 ± 135). Negative control plates containing buffer only were used to assess the spontaneous bacterial mutation rate (typically 15-25 revertants). Toxicity to bacteria was evaluated by microscopic examination of the bacterial background lawn. The amount of bacteria plated, their viability and genotype were routinely tested as described (Maron and Ames, 1983).

4.4. RESULTS

4.4.1. Studies in TA98: To allow the visualization of either an activation or deactivation response the concentration of toxicants was adjusted to give a bacterial response of approximately 500-1000 TA98 revertants per plate . Thus, 5 ng, 2.5 ng and 0.5 ng/plate of 1,3-, 1,6- and 1,8-DNP, respectively were used throughout this study. The effect of increasing concentrations of purified NQOR on the mutagenicity of DNP towards TA98 is shown in figure 4.1.1-3. At the given toxicant concentrations, NQOR was capable of enhancing the mutagenic activity of all three DNP isomers. This increase was linear up to 0.2 µg enzyme/plate (375 units) with each of the toxicants. Dicoumarol (100 µM), a classical inhibitor of NQOR, was found to almost completely inhibit the increase in mutagenicity of DNP by purified NQOR (data not shown). Microscopic examination of the 48 hour plates revealed no toxicity to the bacteria under any of the conditions employed in this study.

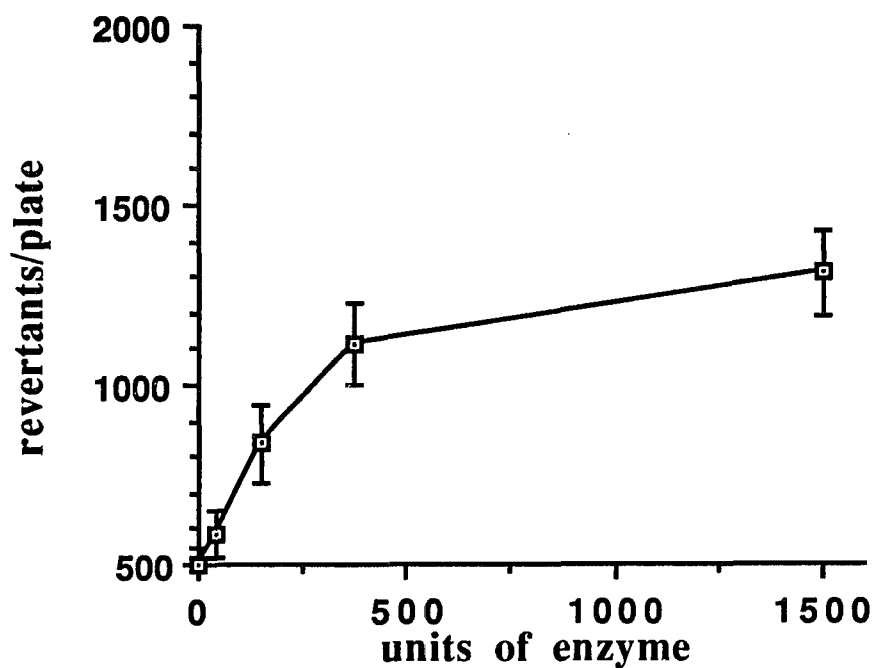


FIGURE 4.1.1. Effect of purified NQOR on the mutagenicity of 1,3-DNP in *S. typhimurium* TA98. NQOR was varied from 0.02-0.8 μg (38-1500 units) in the presence of 5 ng/plate 1,3-DNP. Results are mean \pm SEM of two experiments with triplicate plates. The background revertant rate was subtracted.

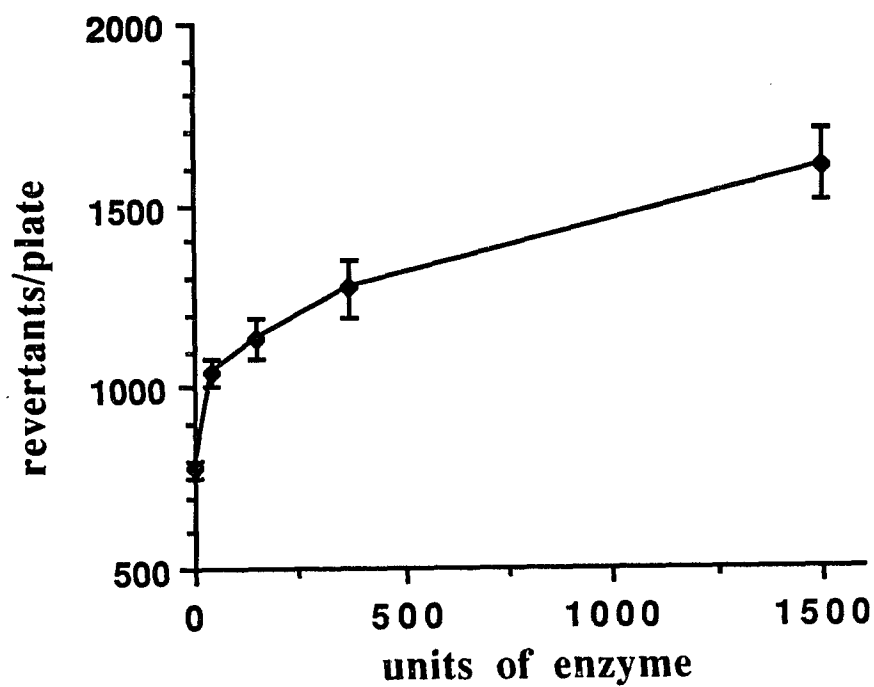


FIGURE 4.1.2. Effect of purified NQOR on the mutagenicity of 1,6-DNP in *S. typhimurium* TA98. NQOR was varied from 0.02-0.8 μ g (38-1500 units) in the presence of 2.5 ng/plate 1,6-DNP. Results are mean \pm SEM of two experiments with triplicate plates. The background revertant rate was subtracted.

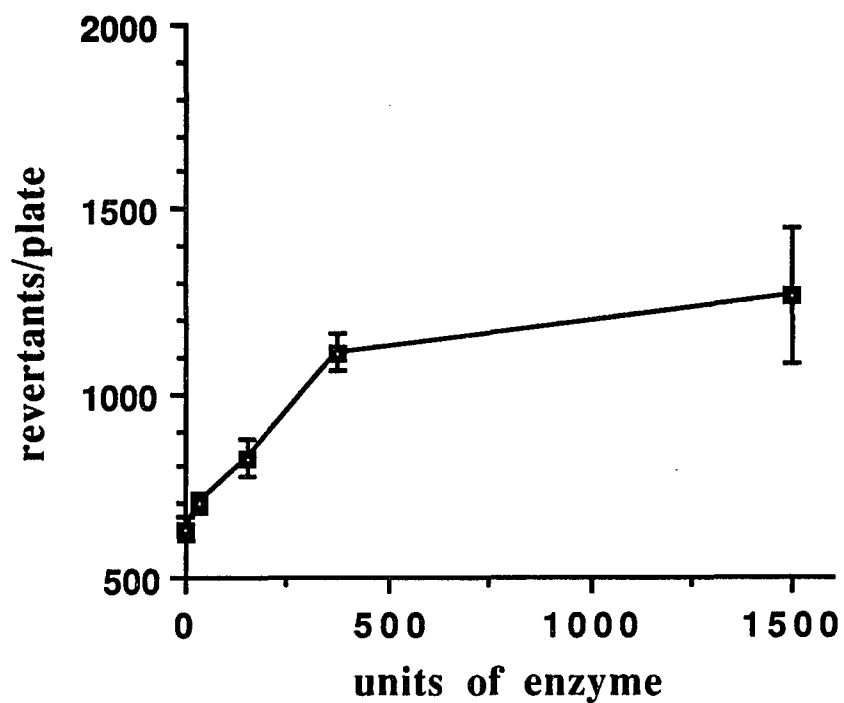


FIGURE 4.1.3. Effect of purified NQOR on the mutagenicity of 1,8-DNP in *S. typhimurium* TA98. NQOR was varied from 0.02-0.8 μ g (38-1500 units) in the presence of 0.5 ng/plate 1,8-DNP. Results are mean \pm SEM of two experiments with triplicate plates. The background revertant rate was subtracted.

4.4.2. Studies in TA98NR and TA98/1,8-DNP₆: To further characterize the effect of NQOR on the mutagenicity of the DNP isomers, studies were conducted with the bacterial strains TA98NR and TA98/1,8-DNP₆. The former lacks the so-called "classical nitroreductase" (Rosenkranz and Mermelstein, 1983, Howard *et al.*, 1987), and the latter is deficient in O-acetyltransferase activity (McCoy *et al.*, 1983). All of these experiments were performed in parallel with TA98 (in the presence and absence of 150 units of NQOR) as a reference control. Consistent with previous reports (Rosenkranz and Mermelstein, 1983, Fifer *et al.*, 1986) the mutagenicity of 1,3-DNP was negligibly expressed in TA98NR, whereas a considerably stronger mutagenic response was observed with 1,6- and 1,8-DNP that paralleled that seen with TA98 (Table 4.1). The increase in the mutagenicity of 1,6- and 1,8-DNP by added NQOR was virtually identical in TA98NR and TA98 (Table 4.1, also compare Figure 4.1.2, 4.1.3 and 4.2). Varying the concentration of NQOR over the range of 0.02-0.8 µg/plate resulted in a proportional increase in the mutagenicity of 1,3-DNP in TA98NR up to 7-fold. At the highest concentration of NQOR tested i.e, 0.8 µg/plate, the number of revertants obtained in TA98NR was similar to that obtained in TA98. This amount of purified enzyme corresponds to the NQOR activity present in approximately 2.5 mg of cytosolic protein from uninduced rat liver or 0.25 mg of cytosolic protein from Aroclor 1254-induced rats. These protein concentrations are typical of cytosolic or S9 fractions employed in the Ames test (Traynor *et al.*, 1988, Traynor *et al.*, 1991, Shah *et al.*, 1990).

In good agreement with the literature (Rosenkranz and Mermelstein, 1983, McCoy *et al.*, 1983, Fifer *et al.*, 1986), the mutagenicity of 1,6-DNP was 85% lower in the acetylase deficient strain, TA98/1,8-DNP₆ as compared to TA98, which contains the full complement of O-acetylase activity. Percentage-wise, the increase in the

mutagenicity of 1,6-DNP catalyzed by NQOR, the only isomer displaying enhanced mutagenicity with NQOR in TA98/1,8-DNP₆, was similar to the increase caused by NQOR in TA98 (Table 4.1, compare Figure 4.1.2 and 4.3). However, the absolute increase in the number of TA98/1,8-DNP₆ revertants due to the presence of NQOR (0.08 µg/plate), was considerably less than in TA98 (39 vs. 462 revertants); respectively (Table 4.1). In contrast, 1,3- and 1,8-DNP did not elicit a mutagenic response in strain TA98/1,8-DNP₆ irrespective of the presence or absence of NQOR (Figure 4.3, Table 4.1).

TABLE 4.1:

Effect of purified NAD(P)H-quinone oxidoreductase on the mutagenicity of DNP in <i>S. typhimurium</i> TA98, TA98NR and TA98/1,8-DNP ₆						
Toxicant	TA98		TA98NR (revertants/plate)		TA98/1,8-DNP ₆	
	-NQOR	+NQOR ^a	-NQOR	+NQOR	-NQOR	+NQOR
1,3-DNP	651 ± 121 ^b	1039 ± 209 (60) ^c	89 ± 26	227 ± 92 (164)	12 ± 0	8 ± 0 (0)
1,6-DNP	824 ± 146	1286 ± 205 (56)	765 ± 67	1464 ± 441 (92)	110 ± 8	149 ± 28 (36)
1,8-DNP	659 ± 3	967 ± 145 (47)	712 ± 69	1321 ± 367 (85)	14 ± 2	7 ± 2 (0)

^a NAD(P)H-quinone oxidoreductase (NQOR) was included at 0.08 µg protein/plate (150 units/plate). 1,3-, 1,6-, and 1,8-DNP concentrations were, respectively, 5, 2.5, and 0.5 ng/plate.

^b Results are the mean ± SEM of triplicate determinations from two experiments. The background reversion rate in presence buffer only was subtracted.

^c Numbers in parentheses refer to the per cent activation as compared to the absence of NQOR.

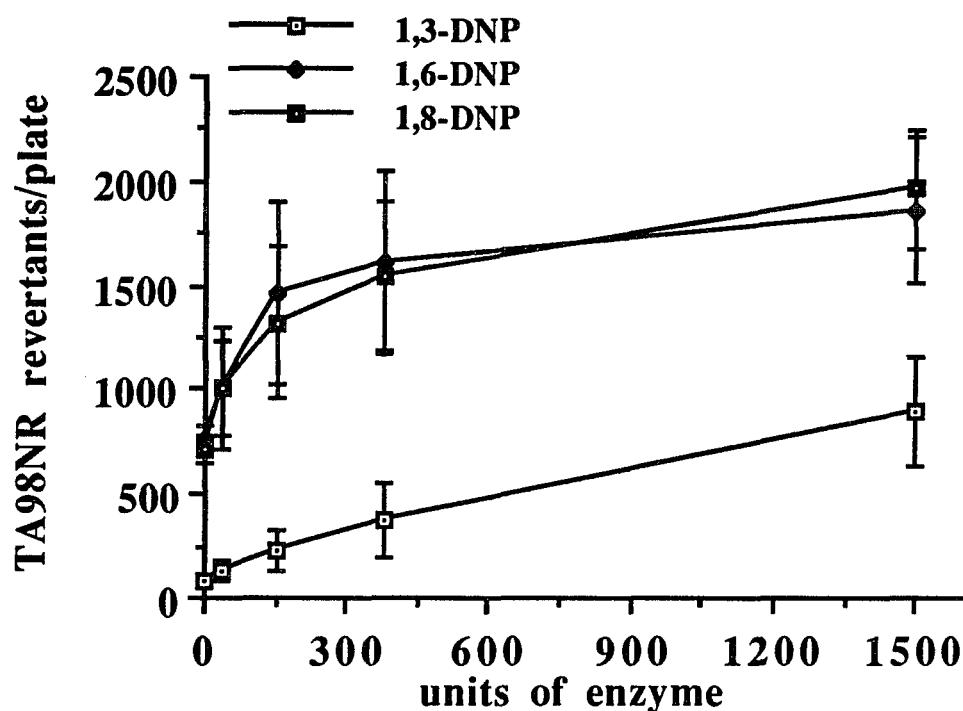


FIGURE 4.2. Mutagenicity of DNP in *S. typhimurium* TA98NR, the nitroreductase-deficient strain at 5, 2.5 and 0.5 ng/plate of 1,3-, 1,6- and 1,8-DNP, respectively. NQOR was added at 0.02-0.8 μ g (38-1500 units/plate). Results are mean \pm SEM of two experiments with triplicate plates. The background revertant rate was subtracted.

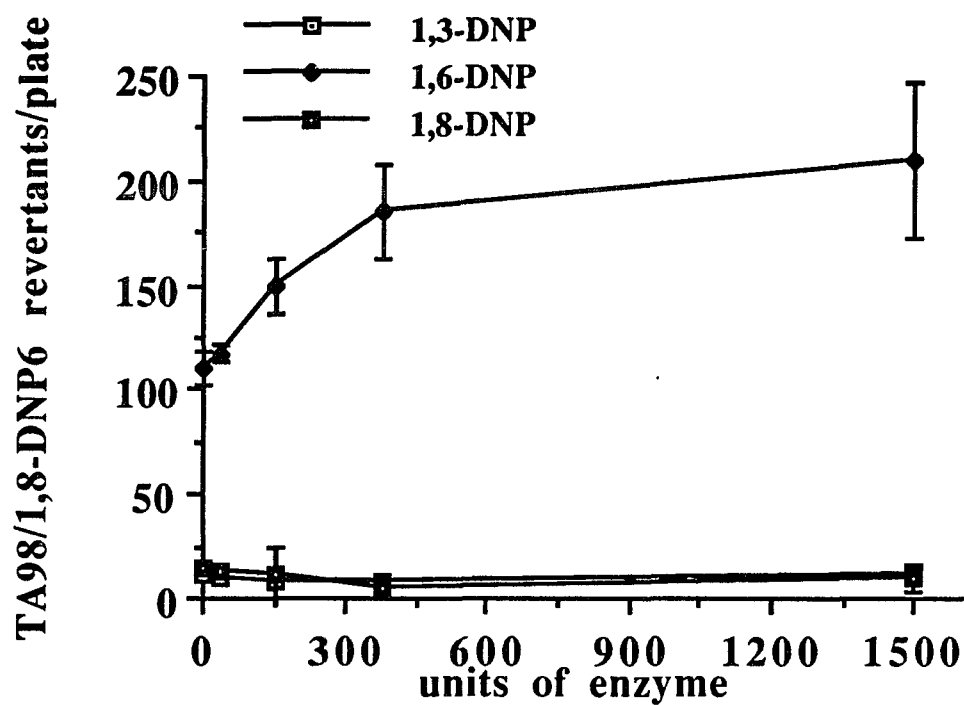


FIGURE 4.3. Mutagenicity of DNP in *S. typhimurium* TA98/1,8-DNP₆, a bacterial strain deficient in O-acetyltransferase activity at 5, 2.5 and 0.5 ng/plate of 1,3-, 1,6- and 1,8-DNP, respectively. NQOR was added at 0.02-0.8 μ g (38-1500 units/plate). Results are mean \pm SEM of two experiments with triplicate plates. The background revertant rate was subtracted.

4.5. Discussion

Herein it is shown that highly purified NQOR causes a concentration-dependent increase in the mutagenicity of 1,3-, 1,6- and 1,8-DNP in *Salmonella typhimurium* TA98. Corroborating the findings of others (Rosenkranz and Mermelstein, 1983), both 1,6- and 1,8-DNP display mutagenicity in TA98NR, whereas the 1,3-substituted isomer is virtually nonmutagenic in this strain. The present study shows that NQOR can substitute for the nitroreductase activity that is deficient in TA98NR. This is evinced by the fact that NQOR (1500 units/plate) was capable of increasing the mutagenic response of 1,3-DNP in TA98NR to the extent that it equaled that obtained in TA98. This finding clearly indicates that NQOR, a two electron-transferring oxidoreductase, is a 1,3-DNP nitroreductase. The mutagenicities of 1,6- and 1,8-DNP were increased in a dose-responsive manner by NQOR in TA98 and TA98NR and no marked differences in the extent of activation were observed between these two strains. The half-wave potential observed upon electrochemical reduction of 1,3-DNP indicates that the first reversible step corresponds to a univalent reduction while that of 1,6- and 1,8-DNP corresponds to a divalent reduction (Howard *et al.*, 1987, Klopman *et al.*, 1984). This finding, coupled with the fact that 1,3-DNP is not mutagenic in TA 98 NR (Rosenkranz and Mermelstein, 1983, Fifer *et al.*, 1986, Howard *et al.*, 1987) led Howard and coworkers to conclude that TA98NR lacks the so-called "classical nitroreductase" which catalyzes the one-electron reduction of 1,3-DNP (Howard *et al.*, 1987). In contrast, activation of 1,6- and 1,8-DNP was proposed to proceed through an initial two-electron transfer step and therefore be independent of this nitroreductase.

One electron reduction of nitroaromatics leads to the formation of reactive radical species such as nitroanion radicals which are capable of both, dismutation and redox

cycling. Dismutation of two nitroanion radicals would result in the formation of one two-electron reduced nitroso species whereas redox cycling would completely regenerate the parent compound (Mason, 1982). NQOR displays nitroreductase (DeFlora *et al.*, 1988, Hajos and Winston, 1990, 1991) and nitroso reductase activity (Horie *et al.*, 1982, Hajos and Winston, 1991). We observed that the increase in the activation of 1,6- and 1,8-DNP by NQOR was similar in TA98NR and TA98 whereas the fold-increase in the activation of 1,3-DNP was much greater in TA98NR. As is true for its activity towards quinone substrates, NQOR is likely to catalyze two-electron reduction of DNP thereby resulting in the formation of the corresponding nitrosonitropyrenes. It is thus implied that NQOR-catalyzed reduction of DNP to the corresponding nitroso compounds constitutes the rate limiting step in the activation of DNP, as indicated by the dependency of the increase in DNP mutagenicity on the concentration of NQOR in the reaction. The possibility, however that NQOR catalyzes the reduction of nitrosonitropyrenes cannot be excluded. In fact, it has been reported that, the bacterial mutagenicity of 1,6- and 1,8-DNP is approximately one order of magnitude greater than that of the corresponding nitrosonitropyrenes (Fifer *et al.*, 1986), an observation which favors the interpretation that NQOR possesses reductase activity towards DNP as well as their two-electron reduced metabolites.

We have previously reported that highly purified NQOR catalyzes nitroreduction of 1,3-, 1,6- and 1,8-DNP with 1,6- and 1,8-DNP being much better substrates for the enzyme than 1,3-DNP (Hajos and Winston, 1990; 1991). Despite the difference in rates of nitroreduction catalyzed by NQOR the extents of the increase in mutagenicity of the three DNP isomers were similar. This might be explained, at least in part, by the fact that the substrate concentrations used in this study were adjusted to result in approximately equal revertant yields. Nevertheless, the potent activation of all three

DNP and particularly that of 1,3-DNP, suggests that neither the rate of nitroreduction of the parent compound nor the valence state of the formed metabolite(s) i.e., odd- or even-electron reduced intermediates, are sufficient in themselves to explain differences in the mutagenic expression of these nitroaromatics. Activation of mutagenic aromatic hydroxylamines by acetylation constitutes a final step in the bioactivation of certain nitroaromatic compounds (Rosenkranz and Mermelstein, 1983, McCoy *et al.*, 1983, Djuric *et al.*, 1985). A mutagenic response was observed in TA98/1,8-DNP₆ with 1,6-DNP, but not with 1,3- or 1,8-DNP. This isomer specificity suggests that 1,6-DNP does not necessarily require acetylation prior to formation of an ultimate DNA-binding species. We reasoned that the use of the acetylase-deficient strain would identify whether NQOR is capable of the formation of DNP metabolites which do not strictly require subsequent acetylation to express their mutagenicity. Only 1,6-DNP showed an increase in the bacterial response upon addition of the enzyme and this increase was responsive to the concentration of NQOR present (Table 4.1, Figure 4.3). The extent of increase in 1,6-DNP mutagenicity in the acetylase-deficient strain was less than that observed with TA98 which is to be expected as acetylation of 1,6-DNP leads to a more mutagenic species (Fifer *et al.*, 1986, Table 4.1).

Rat liver cytosolic NQOR is shown herein to be a mammalian reductase which can compete with bacterial reductases in the nitroreduction of DNP in the Ames test. The enzyme is active towards all three DNP isomers, and at concentrations (activities) comparable to that of rat liver cytosolic fractions commonly employed in the Ames test, significantly increases the mutagenicity of DNP. Our preliminary findings on the cytosolic activation of DNP in the Ames test and the differential effects observed upon pretreatment of the animals with ethanol or Aroclor-1254 (Traynor *et al.*, 1988, Shah *et al.*, 1990) might, at least in part, be ascribed to induction of cytosolic NQOR. The well

known role of NQOR in the removal of redox cycling semi-quinone radicals (Ernster, 1969; Brunmark and Cadenas, 1989) and its potent induction in persistent hepatocyte nodules induced by chemical carcinogenesis (Williams *et al.*, 1986), NQOR has suggested a protective role for this enzyme. However, NQOR also enhances the toxic action of certain quinones such as mitomycin C and diaziquone (Siegel *et al.*, 1990 a;b). Similarly, NQOR can catalyze both the detoxification and toxification of nitroarenes (DeFlora *et al.*, 1988; Knox *et al.*, 1989; this paper)

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Chapter 5

Role of cytosolic NAD(P)H-quinone oxidoreductase and alcohol dehydrogenase in the reduction of p-nitrosophenol following chronic ethanol ingestion

5.1. Abstract: Rats fed an ethanol-containing diet for 4 weeks showed a 3-5 fold increase over isocalorically pair-fed controls with respect to cytosolic NAD(P)H:quinone oxidoreductase (NQOR) [E.C.1.6.99.2] with both menadione and dichlorophenol-indophenol as substrates. Rates of NAD(P)H-dependent p-nitrosophenol (pNSP) reduction catalyzed by rat liver cytosolic fractions were increased 1.5-2 fold upon pretreatment of the animal with ethanol. NADH-dependent C-nitroso reductase activity was inhibited 70-80% by pyrazole and thus can be attributed mainly to alcohol dehydrogenase(s). In contrast, NQOR contributed almost exclusively to the NADPH-dependent C-nitroso reductase activity in cytosol as judged by the almost complete inhibition of the reaction by dicoumarol. Purified rat liver cytosolic NQOR catalyzed the NADH- and NADPH-dependent reduction of pNSP to p-aminophenol, whereas the parent compound, *p*-nitrophenol was not reduced by NQOR. We suggest that the increase in cytosolic NQOR activity following ethanol ingestion enhances the reduction of the C-nitrosoaromatics formed upon cytosolic metabolism of arylamines or nitroarenes.

5.2. Introduction

Chronic ethanol consumption alters the metabolism of numerous xenobiotic and endogenous compounds, a phenomenon generally ascribed to the specific induction of the alcohol-inducible cytochrome P450 isoenzyme (CYP 2E1)¹ (Koop *et al.*, 1982; Lieber, 1990). Induction of this isoenzyme has been linked to the increased oxidation of N-nitrosocompounds such as the chemical mutagens N-nitrosodimethylamine (Tu *et al.*, 1981; Garro *et al.*, 1981) and N-nitrosopyrrolidine (McCoy *et al.*, 1979), to the ring-hydroxylations of the nitroarene p-nitrophenol (Koop, 1986; Reinke and Moyer, 1985) and the aromatic amine aniline (Ko *et al.*, 1987; Winston and Narayan, 1988). The activation of 2-aminofluorene, a benchmark mutagenic arylamine, is lowered by liver microsomal fractions from ethanol-fed rats, but increased by the corresponding cytosolic fraction, as compared to pair-fed controls (Traynor *et al.*, 1991).

Aromatic C-nitroso compounds are common intermediates in the hepatic metabolism of aromatic amines and nitroaromatics. While the activation of mutagenic arylamines depends largely on N-hydroxylation by microsomal mixed function oxygenases, nitroaromatics may be activated via reduction of the nitro moiety by both cytosolic and microsomal fractions. Thus oxidation or reduction processes can result in the formation of hydroxylamino and nitroso compounds, both of which are precursors of the ultimate mutagenic species putatively, the nitrenium ion. We have recently

¹ The nomenclature for cytochrome P450 has undergone several modifications over the past few years. We herein follow the updated nomenclature recommended by Nebert *et al.* (1989). The alcohol-inducible cytochrome P450 isoenzyme CYP 2E1 has also been referred to as cytochrome P-450-ALC., cytochrome P450j, cytochrome P450 LM3a and cytochrome P450IIE1.

reported that NQOR catalyzes nitroreduction of three potently mutagenic dinitropyrenes and that the contribution of this enzyme to the total cytosolic nitroreductase activity is enhanced upon pretreatment of the experimental animal with the polychlorinated biphenyl mixture Aroclor-1254 (Hajos and Winston, 1991). Further, xanthine oxidase has been shown to catalyze the formation of hydroxylaminopyrene from 1-nitropyrene (Howard and Beland, 1982). Studies by Horie *et al.* (1980) have shown that NADH-dependent p-nitrosophenol (pNSP) reductase activity is abundant in cytosolic fractions from various animals. In the rat, organs with high C-nitrosoreductase activity were found to be the liver, the lung, the stomach and the heart. Two decades ago, Dunn and Bernhard (1971) showed that purified horse liver alcohol dehydrogenase (ADH) could catalyze the reduction of p-nitroso-N,N-dimethylaniline. Because the nitroso moiety is isosteric and isoelectronic with the aldehyde functionality, the investigators employed this substrate as an analog of aromatic aldehydes, which are known substrates for ADH, in order to study the kinetic mechanism of the enzyme. As the liver was the only organ which showed both C-nitrosoreductase activity toward pNSP and aldehyde reductase activity, Horie *et al.* (1980) concluded that the major enzyme catalyzing the reduction of pNSP is probably ADH, but that other enzymes must exist which also can reduce this compound. The identity of ADH and the hepatic C-nitroso reductase was further supported by copurification of NADH-dependent aldehyde- and pNSP-reductase activity from porcine liver (Kuwada *et al.*, 1980). The resulting partially purified preparation resembled the characteristics of ADH. Subsequent studies showed that C-nitrosoreductase activity in porcine heart coeluted with menadione reductase activity and was supported by either NADH or NADPH (Horie and Ogura, 1980). A further purification yielded an enzyme fraction that was judged to be approximately 90% pure and shared many characteristics of NQOR, albeit some differences were noted (Horie *et*

al., 1982). Surprisingly, the toxicological and pharmacological significance of the C-nitroso reductase activities of either ADH or NQOR have received little, if any attention. These activities deserve consideration, as ADH catalyzes the NADH-dependent reduction of pNSP as well as of p-nitroso-N,N-dimethylaniline (Horie *et al.*, 1980; Dunn and Bernhard, 1971). NQOR, which can catalyze the NADH- and NADPH-dependent reduction of its substrates, and is highly inducible in animals following pretreatment with various xenobiotic chemicals, has been shown to reduce the nitrosoaromatics pNSP, nitrosobenzene, α -nitroso- β -naphthol, β -nitroso- α -naphthol, and 5-nitroso-8-quinolinol (Horie *et al.*, 1982).

The apparent importance of these cytosolic activities in the mutagenic expression of arylamines and nitroarenes, coupled with the known effects of ethanol consumption as a modulator of these activities, prompted the present investigation of the effects of ethanol ingestion on cytosol-mediated metabolism of the aromatic C-nitroso compound, *p*-nitrosophenol. In this regard it should be noted that *p*-nitrosophenol is the two-electron reduced form of *p*-nitrophenol, the substrate for microsomal *p*-nitrophenol hydroxylase, as well as the four-electron oxidized form of *p*-aminophenol, the product of the aniline hydroxylase reaction. Both of these hydroxylase activities are highly inducible upon ethanol ingestion and are preferentially catalyzed by CYP 2E1, the ethanol-inducible isoform of cytochrome P450 (Reinke and Moyer, 1985; Koop, 1986, Ko *et al.*, 1987; Winston and Narayan, 1988).

5.3. Experimental procedures

5.3.1. Chemicals and enzymes. Allopurinol, cibacron blue 3GA-agarose, cibacron blue 3GA, dicoumarol, ferricytochrome c, hypoxanthine, xanthine, pyrazole, menadione, dichlorophenol-indophenol, NADH, NADPH, potassium ferricyanide, Tris-base and Tris-Cl, were from Sigma (St. Louis, MO). p-Aminophenol, p-nitrosophenol, p-nitrophenol, KCN and phenol were from Aldrich Chemical Company, (St. Louis, MO).

5.3.2. Animals and pretreatment Male Sprague-Dawley rats (approximately 100 g) from Hilltop Animals Inc. (Scottsdale, PA) were maintained in separate cages on a 12-hour light/dark cycle in a temperature and humidity-controlled environment and fed a diet of Purina rat chow *ad libitum* until they reached a weight of 150-200 g. At this point a 30 day liquid diet (Bioserve Inc., Frenchtown, NJ) was employed which contained ethanol as 36% of the total calories (Lieber and DeCarli, 1982). Control animals were pair-fed isocalorically with carbohydrate substituted for ethanol. Rats were killed by decapitation, the livers removed and liver fractions prepared by differential centrifugation as described previously (Lake, 1987). The 105,000 x g supernatant was taken as cytosol. Tissue fractions were stored at -70°C prior to use.

5.3.3. Enzyme assays: Formation of p-aminophenol from p-nitrosophenol (pNSP) was determined by a modification of the procedure described in (Horie *et al.*, 1980). Shortly, 200 µM pNSP was incubated for a specified period of time in the presence of 200 µM NADH or NADPH and an appropriate amount of activating enzyme (approximately 0.05 mg cytosolic protein or 0.4 µg purified NQOR) in 50 mM

potassium phosphate buffer, pH 7.4, in a final volume of 2 ml. The reaction was terminated by addition of 300 μ l of 1 M Na_2CO_3 and p-aminophenol was detected spectrophotometrically by subsequent addition of 300 μ l of 5% (w/v) aqueous phenol and 200 μ l of 0.2% (w/v) potassium ferricyanide. The color was allowed to develop for at least 15 minutes and the absorbance read at 630 nm against a blank containing all reagents but the enzyme fraction. The concentration of p-aminophenol formed was calculated from a standard curve using commercially available p-aminophenol. The reaction was linear with respect to protein concentration and incubation time. Additional incubations contained 5-200 μ M dicoumarol or 0.1-10 mM pyrazole. Control experiments established that at the inhibitor concentrations employed, dicoumarol did not inhibit alcohol dehydrogenase-catalyzed ethanol oxidation and pyrazole had no effect on NQOR-catalyzed reduction of menadione.

Xanthine oxidase-catalyzed reduction of pNSP was determined with 1 mM hypoxanthine as cofactor in the presence or absence of 10 μ M allopurinol. NADH-dependent C-nitrosoreductase activity of purified NQOR was further determined by following the oxidation of NADH spectrophotometrically at 340 nm in the presence of the substrate pNSP and the concomitant loss of absorbance at 410 nm due to reduction of pNSP. NQOR activity was determined as described by Ernster (1969) using 40 μ M dichlorophenol-indophenol (DCPIP) or 20 μ M menadione as substrate in 50 mM Tris-Cl, pH 7.4 containing and 50 μ M ferricytochrome c (with menadione as the substrate), 0.2 % (v/v) Tween-20, 0.5 mM KCN and either 200 μ M NADH or 200 μ M NADPH. The dicoumarol-sensitive portion of the reaction was taken as NQOR activity. Alcohol dehydrogenase activity was determined by following the change in absorbance at 340 nm due to the reduction of NADH, 14 mM ethanol was employed as substrate in 50 mM potassium phosphate, pH 7.4. Xanthine oxidase activity was determined as outlined by

Ragolapajan (1985) by following the formation of urate at 295 nm. Protein was determined according to the method of Bradford (1976) using bovine serum albumin as a reference standard.

5.3.4. Enzyme preparation: Rat liver cytosolic NAD(P)H:quinone oxidoreductase (E.C.1.6.99.2) was purified to electrophoretic homogeneity from rat liver cytosol by the method of Prochaska (1988).

5.4. Results

5.4.1. Cytosolic NAD(P)H-quinone oxidoreductase, xanthine oxidase and alcohol dehydrogenase activities . Compared to pair-fed control animals ethanol-fed rats showed nearly 3-fold and 5-fold increases in cytosolic NQOR activity with dichlorophenol-indophenol (DCPIP) and menadione as substrates, respectively (Table 5.1A). Dicoumarol inhibited the NAD(P)H-dependent reduction of both substrates by at least 90%, independent of the source of cytosol employed (not shown). Cytosolic NQOR activities were approximately 5-fold lower in rats maintained on a pair-fed control liquid-diet as compared to those fed a standard laboratory chow diet. In contrast, activities of the cytosolic marker enzyme glucose-6-phosphate dehydrogenase were found to be identical in cytosol from pair-fed and chow-fed rats and slightly elevated in that from ethanol-fed rats (data not shown). NQOR is known to be inducible by dietary antioxidants contained in laboratory chow (Williams *et al.*, 1986, Talalay *et al.*, 1988) thus, the differences in the basal NQOR activities of rats fed a pair-fed liquid diet versus those fed standard rat chow may well reflect the differences in the antioxidant content of the diet. The data in Table 5.1B show that cytosol from ethanol-fed rats displays a small, and statistically insignificant increase in alcohol dehydrogenase (ADH) activity as compared to pair-fed or chow-fed controls. Whereas increased ADH activity has been reported in some earlier studies, the present results showing a lack of a significant increase of ADH activity upon chronic ethanol consumption is consistent with the preponderance of the literature (see Khanna and Israel, 1980 for review). Xanthine oxidase activity was significantly elevated in cytosol from ethanol-fed rats, albeit a rather large biological variance was seen within each treatment group (Table 5.1B).

TABLE 5.1

(A) NAD(P)H-menadione and DCPIP oxidoreductase activity:

Treatment:	cofactor	nmoles DCPIP reduced min x mg protein	nmoles Cyt. c reduced ^a min x mg protein
Pair-fed	NADPH	39 ± 1 ^{b,c}	73 ± 14 ^{b,c}
	NADH	36 ± 6	68 ± 10
Ethanol-fed	NADPH	128 ± 1 (3.3) ^d	309 ± 20 (4.2)
	NADH	114 ± 10 (3.2)	319 ± 14 (4.7)
Chow-fed	NADPH	221 ± 10 ^b	362 ± 14
	NADH	188 ± 4	373 ± 45

B) Alcohol dehydrogenase and xanthine oxidase activity:

	nmol NADH formed min x mg protein	units xanthine oxidase mg protein
Pair-fed	5.1 ± 1.0 ^b	9.7 ± 3.8 ^e
Ethanol-fed	6.4 ± 1.4	21.8 ± 16.9
Chow-fed	5.3 ± 0.3	N.D. ^f

^a Incubations were performed in the presence of 20 µM menadione using 50 µM cytochrome c as the terminal electron acceptor. Further details are described in Materials and methods.

^b Results are the mean ± SD of at least triplicate determinations with pooled cytosol from 3-6 rats.

^c Rates represent dicoumarol-uninhibited minus -inhibited rate.

^d Numbers given in parentheses are the fold-induction as compared to pair-fed controls.

^e The allopurinol-sensitive portion of the rate is shown. Results are the mean ± SD of single determinations with 6 cytosolic samples from each group. One unit of xanthine oxidase is defined as the amount of enzyme which causes a change in optical density of OD=1.0 at 295 nm in presence of 50 µM hypoxanthine.

^f N.D.; not determined.

5.4.2. Reduction of *p*-nitrosophenol by rat liver cytosol. NADH-dependent reduction of pNSP was increased by approximately 50% in cytosol from ethanol-fed rats (closed symbols) as compared to pair-fed controls (open symbols) over the range of substrate concentrations studied (Figure 5.1, squares). Since inhibition was observed at pNSP concentrations above 200 μ M all subsequent experiments with cytosol from both sources employed 200 μ M substrate. The effect of increasing concentrations of pyrazole and dicoumarol on the cytosolic reduction of pNSP is shown in Figure 5.2. These compounds are classical inhibitors of NQOR and alcohol dehydrogenase, respectively. It is noted that pyrazole potently inhibited NADH-dependent C-nitrosoreductase activity in cytosol from both ethanol- and pair-fed rats (Figure 5.2a, squares). In contrast, dicoumarol was found to be only slightly inhibitory over the range of inhibitor concentrations tested (Figure 5.2b, squares). Additional experiments involved the use of allopurinol to inhibit cytosolic xanthine dehydrogenase/oxidase activity in the presence of NADH or hypoxanthine as cofactor. At concentrations of inhibitor that completely inhibited urate formation from xanthine with either cytosol or purified xanthine oxidase, allopurinol was found to have no effect on the NADH-dependent rate of reduction of pNSP in cytosol from either source (data not shown). Further, cytosolic xanthine oxidase does not appear to catalyze the hypoxanthine-dependent reduction of pNSP as judged from the lack of formation of pAP from pNSP by cytosol from either ethanol- or pair-fed rats in the presence of 1 mM hypoxanthine. Purified xanthine oxidase in the presence of hypoxanthine (at enzyme concentrations that readily catalyzed the formation of urate from hypoxanthine) did not affect an absorbance change at 410 nm, a wavelength at which pNSP, but not pAP strongly absorbs (data not shown).

The effect of increasing substrate concentrations on the NADPH-dependent cytosolic reduction of pNSP was investigated as a function of ethanol feeding (Figure 5.1, circles). Saturation was achieved at 200 μ M pNSP and substrate inhibition was seen above this concentration in both cytosolic systems. Cytosolic NADPH-dependent C-nitrosoreductase activity was considerably lower than the corresponding NADH-dependent activity. Ethanol ingestion significantly increased the NADPH-dependent C-nitrosoreductase activity over the entire range of substrate concentrations studied (Figure 5.1, circles) and pyrazole (0.1-5 mM) did not inhibit this activity (Figure 5.2a, circles). Virtually complete inhibition of cytosolic ADH activity was seen above 1 mM pyrazole with ethanol as the substrate (data not shown). In contrast to the lack of effect of pyrazole, pNSP-reduction was strongly inhibited by dicoumarol; almost complete inhibition was seen at the higher inhibitor concentrations i.e., above 10 μ M dicoumarol (Figure 5.2b, circles). Furthermore, the percent of inhibition of cytosolic C-nitrosoreductase activity by dicoumarol was greater in ethanol-fed as compared to pair-fed rats. Thus, all of the induced activity was dicoumarol-sensitive as the residual activities were identical.

Cytosolic C-nitrosoreductase activities were determined with each cofactor in the presence and absence of dicoumarol or pyrazole at the optimized substrate and inhibitor concentrations (Table 5.2). The data verify the increase in C-nitrosoreductase activity by chronic ethanol consumption. While NQOR can utilize NADH or NADPH as cofactors, the C-nitrosoreductase activity of ADH is quite specific for NADH. Thus it was anticipated that the contributions of NQOR to the NADPH-dependent reduction of pNSP would be greater than to the NADH-dependent activity. As shown in Table 5.2, dicoumarol inhibited the NADPH-dependent reduction of pNSP by cytosol from ethanol- and pair-fed rats to 78% and 65%, respectively. The dicoumarol-sensitive rate

of reduction was 5.6 nmol pAP formed/min/mg protein in cytosol from ethanol-fed and 2.8 nmol pAP formed/min/mg protein in cytosol from pair-fed animals. The inhibition by pyrazole was 13% and 8%, respectively. This corresponds to only 12% and 5% of the NADH-dependent rate of pNSP reduction being inhibitable by pyrazole. In turn, the NADPH-dependent, dicoumarol-sensitive rate of the reaction was 2.4- and 2.7-fold higher than the NADH-linked activity. Induction of cytosolic NQOR activity thus increases cytosolic C-nitrosoreductase activity and the effects are more pronounced when NADPH is available as the cofactor probably owing to the fact that there is little competition with cytosolic ADH.

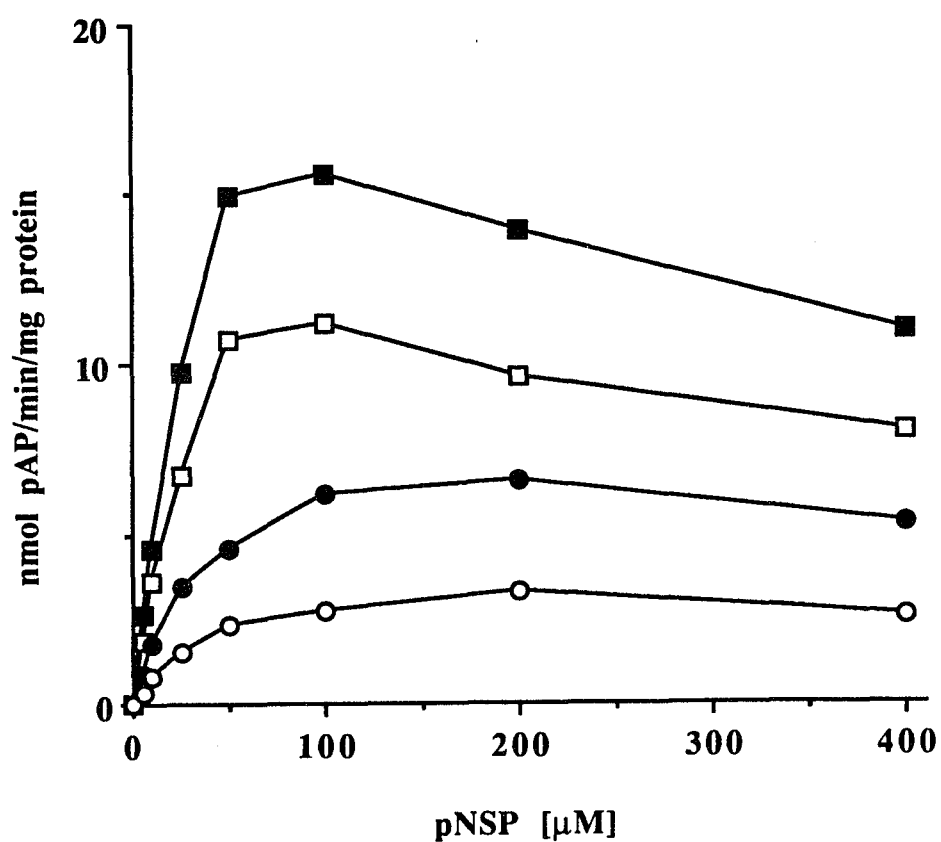


Fig. 5.1. NAD(P)H-dependent C-nitrosoreductase activity in cytosol from ethanol- and pair-fed rats: Effect of substrate concentration. Cytosol from ethanol-fed rats (closed symbols) or pair-fed controls (open symbols) was incubated in the presence of increasing concentrations of pNSP as described in Materials and Methods. Rates for the NADH-dependent (squares) and NADPH-dependent (circles) C-nitrosoreductase activities are shown. Results are the mean of duplicate determinations at each substrate concentration with pooled cytosol from six animals in each treatment group.

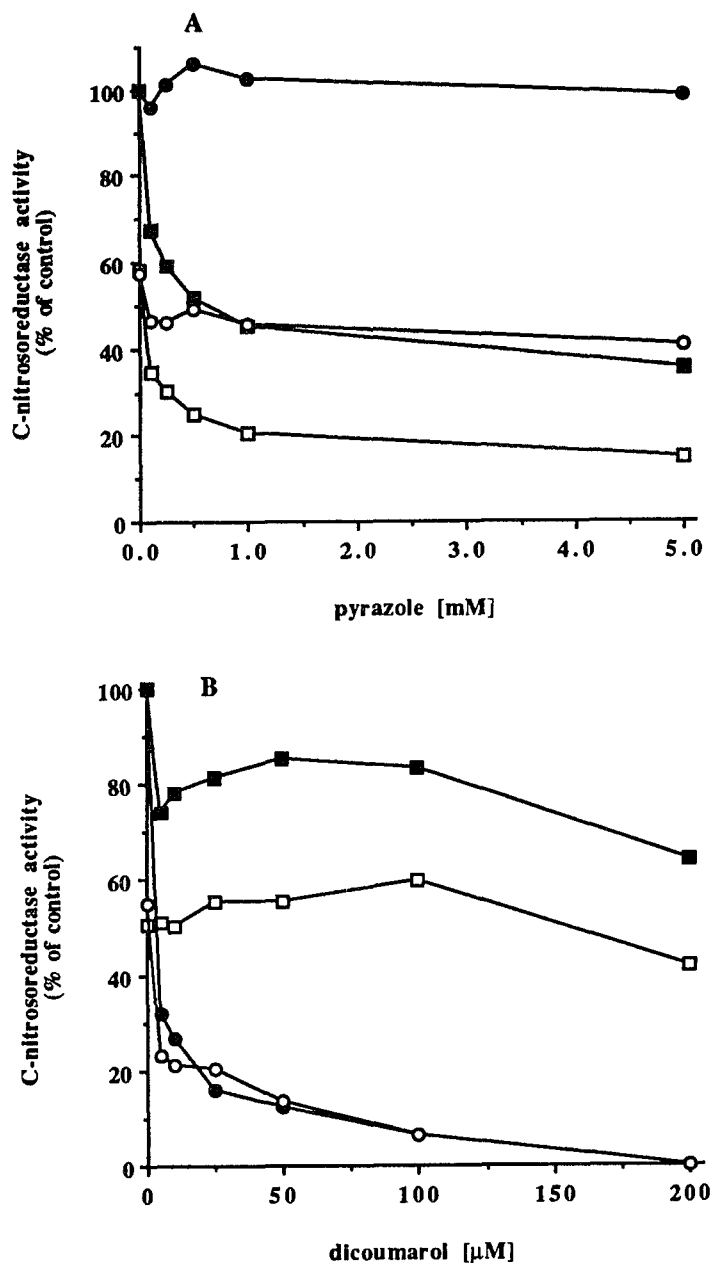


Fig. 5.2. NAD(P)H-dependent C-nitrosoreductase activity in cytosol from ethanol- and pair-fed rats: Effect of A) pyrazole and B) dicoumarol. Cytosol from ethanol-fed rats (closed symbols) or pair-fed controls (open symbols) was incubated in the presence of increasing concentrations of inhibitors as described in Materials and Methods. Rates for the NADH-dependent (squares) and NADPH-dependent (circles) C-nitrosoreductase activities are shown as per cent activity relative to the C-nitrosoreductase activity in cytosol from ethanol-fed rats in the absence of inhibitor. Results are the mean of duplicate determinations at each inhibitor concentration with pooled cytosol from six animals in each treatment group.

TABLE 5.2. NAD(P)H-DEPENDENT NITROSOREDUCTION OF p-NITROSOPHENOL IN RAT LIVER CYTOSOL FROM ETHANOL- AND PAIR-FED RATS

Treatment:		control	+ 100 μ M DC	+ 1mM Pyrazole
Pair-fed	NADH	6.9 \pm 1.8 ^{a, b}	5.8 \pm 1.8 ^{**} (15 %) ^d	1.3 \pm 1.2 ^{**} (81 %) ^d
	NADPH	4.2 \pm 1.2	1.5 \pm 1.3 ^{**} (65 %)	4.0 \pm 1.2 [†] (8%)
Ethanol-fed	NADH	11.4 \pm 2.1 [*] (66 %) ^c	9.1 \pm 1.8 ^{**} (20 %)	3.6 \pm 2.0 ^{**} (69 %)
	NADPH	7.2 \pm 1.2 [§] (69 %) ^c	1.6 \pm 1.1 ^{**} (78 %)	6.3 \pm 0.1 ^{**} (13 %)

^a The rate is expressed as nmol p-aminophenol formed x min⁻¹ x mg prot.⁻¹

^b Results are mean \pm SD of two or more determinations of at least two experiments with 2 independent preparations of pooled cytosol from 6 rats in each treatment group.

^c Numbers in parentheses refer to the induction of nitrosoreductase activity as compared to pair-fed control.

^d Numbers in parentheses refer to the per cent inhibition by dicoumarol (DC) and pyrazole, respectively

^{*} Different from Pair-fed p<0.001

^{**} Different from absence of inhibitor p<0.0001

[§] Different from Pair-fed p<0.0001

[†] Different from absence of inhibitor p<0.005

5.4.3. Dependence of *p*-nitrosophenol reduction on pH. The pH-dependence of an enzyme-substrate couple is an individual characteristic of enzyme catalyzed reactions and often provides information as to the mechanism of catalysis. Studies in porcine heart cytosol (Horie and Ogura, 1980) have indicated that a NAD(P)H-dependent C-nitrosoreductase, which is probably identical with NQOR, showed a pH-optimum of pH 5.5. This was in noted contrast to the pH-profile obtained with NQOR purified from bovine liver cytosol, which displayed two pH-optima at pH 5.7 and pH 8.5 with menadione as the substrate (Maerki and Martius, 1960). We therefore investigated the pH-dependence of the cytosolic reduction of pNSP as a function of pretreatment and cofactor requirement to further differentiate between ADH- and NQOR-catalyzed C-nitrosoreductase activities. NADH-dependent C-nitrosoreductase displayed a pH-optimum around pH 6.0-6.5 in the ethanol- and pair-fed cytosolic systems and cytosol from ethanol-fed rats was generally more efficient in catalyzing the reduction of pNSP, especially between the pH range of 6.0-7.0 (Figure 5.3a). The pH-dependence of cytosolic NADPH-dependent C-nitrosoreductase activity is shown in Figure 5.3b. A pH-optimum of pH 6.5 was observed, that paralleled that of the NADH-dependent activity. Further, cytosol from ethanol-fed rats was more efficient in catalyzing NADPH-supported pNSP reduction than was cytosol from pair-fed rats in the range of pH 5.5-7.5. It should be noted that cytosolic NADPH-dependent C-nitrosoreductase activity appeared to display a sharper pH-optimum than did the NADH-dependent activities and that rates of reduction of pNSP below pH 7.0 were substantially higher with NADPH (compare Figure 5.3a and b).

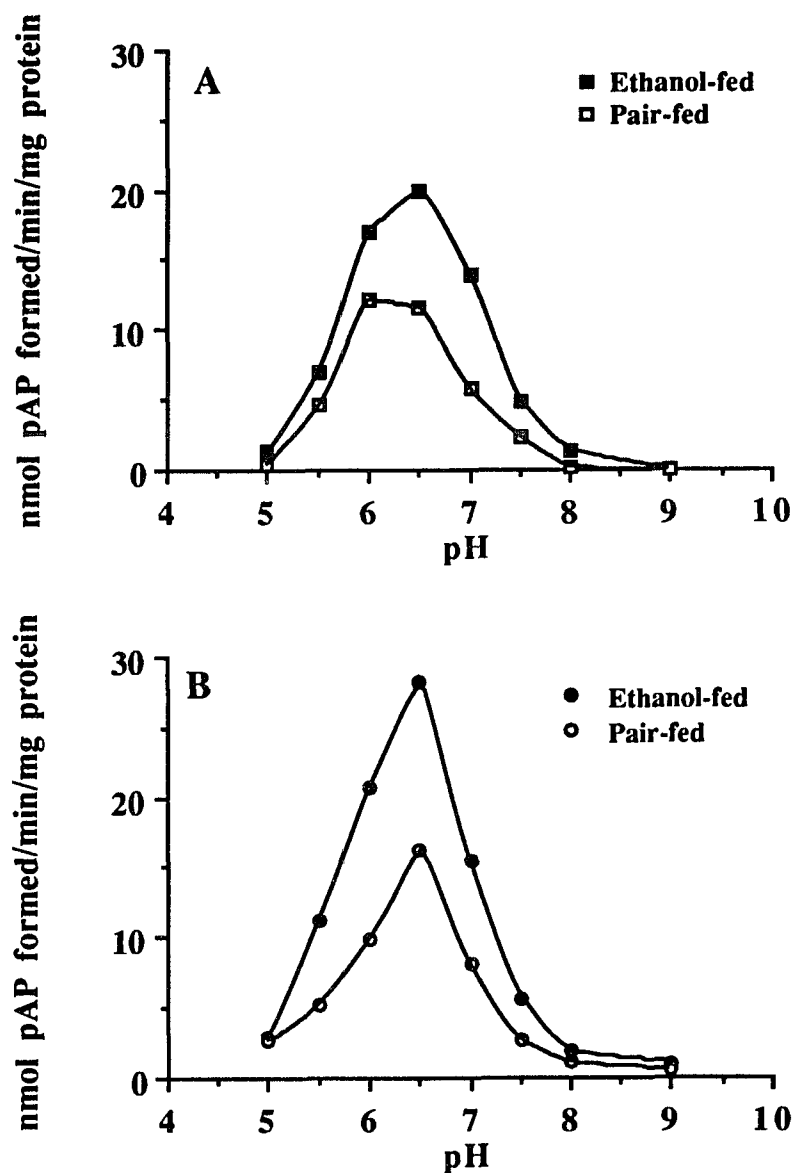


Fig. 5.3. NAD(P)H-dependent C-nitrosoreductase activity in cytosol from ethanol- and pair-fed rats: Dependence on pH. Cytosol from ethanol-fed rats (closed symbols) or pair-fed controls (open symbols) was incubated in the presence of 200 μ M pNSP in the pH range of pH 5.0-9.0 as described in Materials and Methods. Rates for the A) NADH-dependent and B) NADPH-dependent C-nitrosoreductase activities are shown. Results are the mean of duplicate determinations with pooled cytosol from six animals in each treatment group.

5.4.4. C-nitrosoreductase activity of purified rat liver NQOR. Figure 5.4 is a plot of the spectral changes observed upon NADH-dependent reduction of pNSP by purified rat liver NQOR. A loss of absorbance at 340 nm due to oxidation of NADH is paralleled by the disappearance of a peak at 410 nm due to reduction of the nitrosocompound to pAP. Purified NQOR did not catalyze any detectable NAD(P)H-dependent reduction of the parent compound p-nitrophenol (data not shown).

The catalytic activity of purified NQOR towards pNSP was further substantiated by following the NAD(P)H-dependent appearance of pAP employing chromophore formation upon addition of phenol under alkaline conditions (Horie *et al.*, 1980). Similar to results obtained with cytosolic fractions, purified NQOR displayed substrate saturation at about 200 μ M however, in contrast to cytosol, no substrate inhibition was observed even at 400 μ M pNSP (Figure 5.5). It is therefore unlikely that the inhibition observed in cytosol was due to the inhibition of NQOR at high concentrations of pNSP employed. Furthermore, the rates of NADPH-dependent reduction of pNSP was found to be greater than those of the NADH-dependent reduction at all substrate concentrations tested, a finding that was in direct contrast to that with the same activity measured in cytosol.

The pH-profile of purified NQOR from bovine liver with menadione as the substrate displays two pH maxima at pH 5.7 and pH 8.5, while only the maximum at pH 5.7 remains in the presence of cytochrome c as the terminal electron acceptor (Maerki and Martius, 1960). In contrast, one pH maximum at pH 5.5 was found for NADH:pNSP nitrosoreductase activity in porcine heart cytosol and at pH 6.0-6.5 for NAD(P)H-dependent reduction of pNSP in rat liver cytosol (Figure 5.3). Because of these discrepancies, we have investigated the pH profile for pNSP reduction by highly purified NQOR. The NADH- and NADPH-dependent rates of reduction of pNSP by

purified NQOR is virtually identical above pH 7.5 whereas, NADPH is about twice as effective as a cofactor than NADH below the optimum pH (approximately pH 6.5) (Figure 5.6). The pH-optimum for pNSP-reduction by purified NQOR coincides therefore, with the cytosolic optimum.

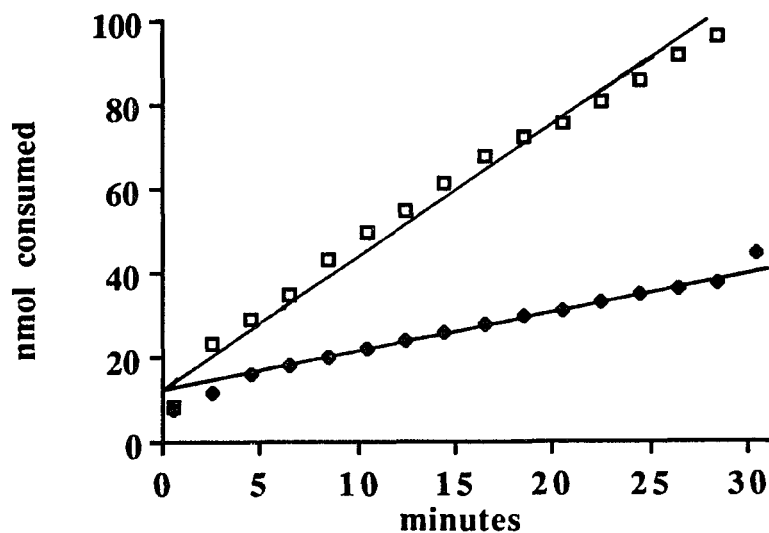


Fig. 5.4. NADH-dependent C-nitrosoreductase activity of purified NQOR: Dependence on time. Purified NQOR (approximately 0.4 $\mu\text{g/ml}$) was incubated in the presence of 200 μM pNSP as described in Materials and Methods. The change in absorbance at 340 nm (squares, expressed as nmol NADH oxidized) and at 410 nm (diamonds, expressed as nmol pNSP reduced) is shown as a function of time.

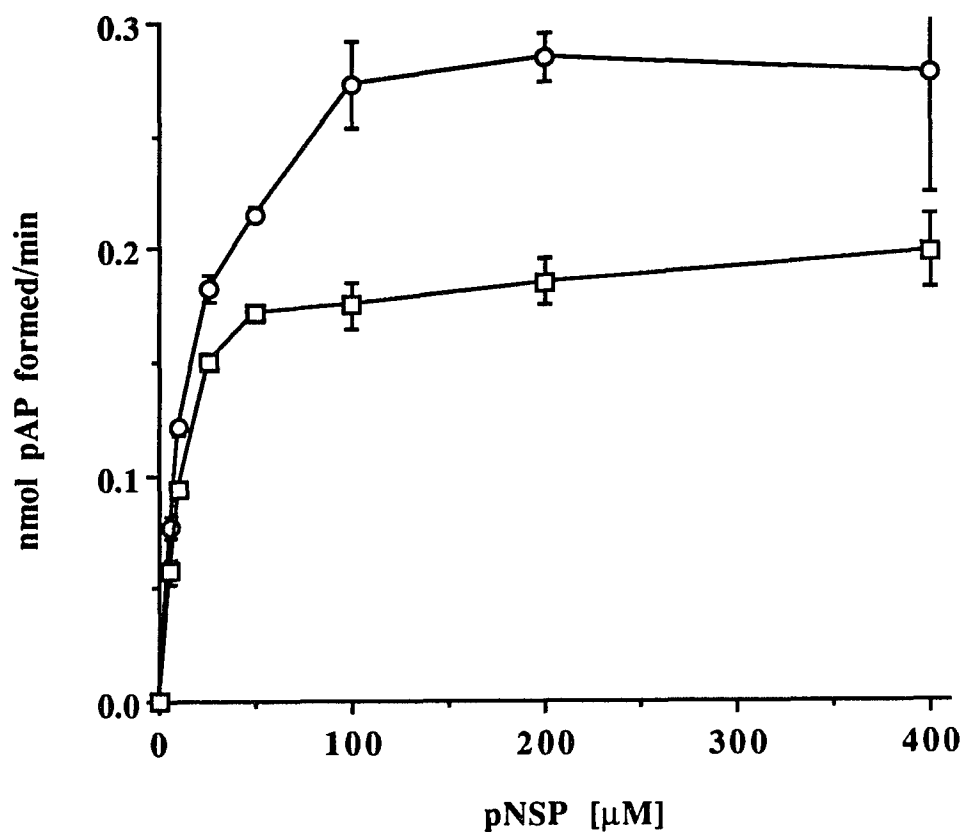


Fig. 5.5. NAD(P)H-dependent C-nitrosoreductase activity of purified NQOR: Effect of substrate concentration. Purified NQOR (approximately 0.4 $\mu\text{g/ml}$) was incubated in the presence of increasing concentrations of pNSP as described in Materials and Methods. Rates for the NADH-dependent (squares) and NADPH-dependent (circles) C-nitrosoreductase activities are shown. Results are the mean \pm SD of triplicate determinations at each substrate concentration.

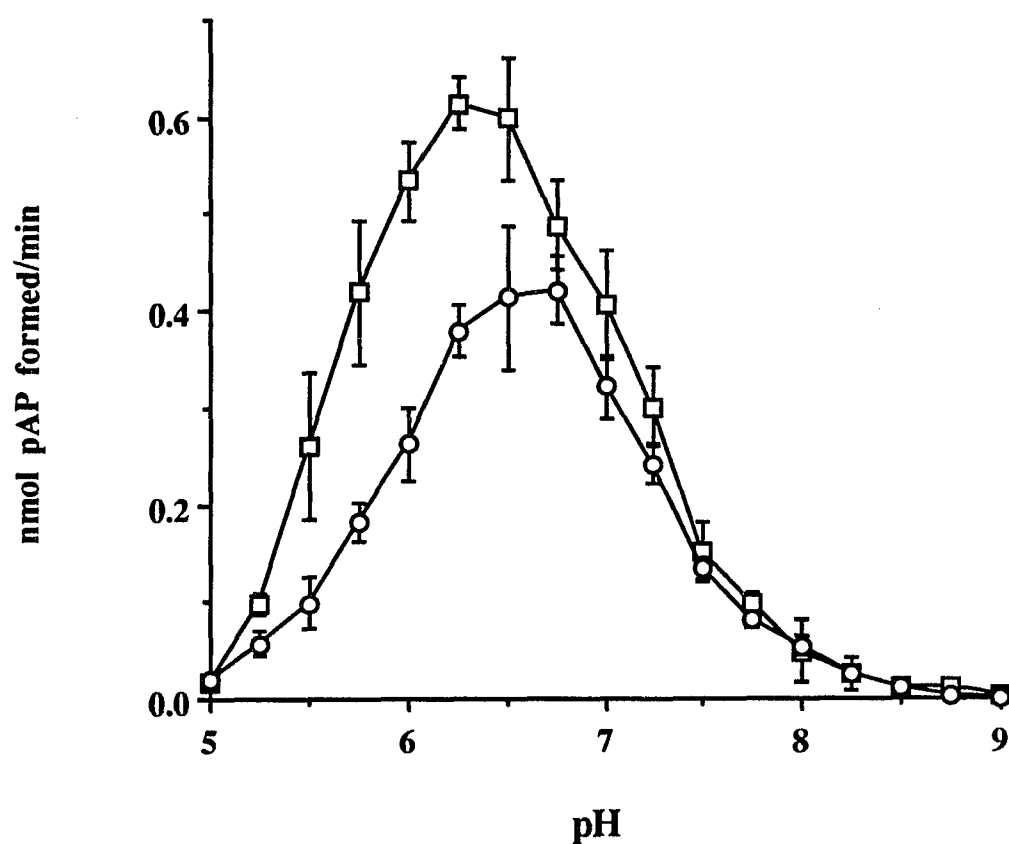


Fig. 5.6. NAD(P)H-dependent C-nitrosoreductase activity of purified NQOR: Effect of pH. Purified NQOR (approximately 0.4 $\mu\text{g/ml}$) was incubated in the presence of 200 μM pNSP in the pH range of pH 5.0-9.0 as described in Materials and Methods. Rates for the NADH-dependent (squares) and NADPH-dependent (circles) C-nitrosoreductase activities are shown. Results are the mean \pm SD of four determinations for each data point.

5.5. Discussion

Previous studies (Wilcox and Parry, 1981; Djuric *et al.*, 1986; Shah *et al.*, 1990; Traynor *et al.*, 1991) have shown that both microsomal and cytosolic enzyme activities are involved in the metabolism of mutagenic nitroaromatics and arylamines. Activation of nitroaromatic compounds involves formation of partially reduced metabolites via nitroreduction such as C-nitroso- or hydroxylamino compounds. These metabolites which are capable of interacting with cell macromolecules such as DNA also may be formed by microsomal N-hydroxylation of arylamines, followed by spontaneous autooxidation in the presence of molecular oxygen, or by enzymatic oxidation, for example by certain peroxidases (Boyd and Eling, 1984).

Formation and removal of the nitroso intermediate of the respective parent compound is of the utmost importance in the mutagenic expression of nitroaromatics and arylamines (see introduction). Previous studies have shown that the reduction of aromatic C-nitroso compounds by cytosol and purified enzyme fractions from porcine heart and liver involves NQOR and ADH (Horie *et al.*, 1980; Kuwada *et al.*, 1980, Horie and Ogura, 1980; Horie *et al.*, 1982). Herein we show evidence for the induction of rat liver cytosolic NQOR by ethanol feeding and discuss its consequences in the metabolism of C-nitrosoaromatics. Emphasis was placed on the relative contributions of NQOR and ADH, and their respective cofactor requirements, as a function of ethanol ingestion. Induction of liver cytosolic NQOR was reflected in 1.5- and 2-fold increases in NADH- and NADPH-dependent pNSP nitroso reductase activities, respectively. Neither cytosolic xanthine oxidase/xanthine dehydrogenase nor purified xanthine oxidase appeared to catalyze the reduction of pNSP. At liver physiological pH the majority of the NADH-dependent C-nitroso reductase activity could be attributed to

cytosolic ADH, as judged from the inhibition of the reaction by pyrazole. It is realized, that this is not necessarily a sufficient criterion to invoke ADH in the metabolism of pNSP. The displayed cofactor specificity and the reported C-nitroso reductase activity of horse- or porcine liver ADH however should suffice to positively identify this enzyme (Dunn and Bernhard, 1971; Kuwada *et al.*, 1980; Horie *et al.*, 1982). One might speculate, that upon ethanol ingestion, i.e. in the presence of ethanol, competition between this ADH substrate and pNSP might occur. This would probably lead to a greater participation of NQOR in the NADH-dependent cytosolic reduction of pNSP. In contrast to NADH-linked activities, 78% and 65% of the NADPH-dependent activity was inhibitable by dicoumarol in cytosol from ethanol- and pair-fed animals, respectively. Thus, the major portion of the NADPH-dependent reductase activity is catalyzed by NQOR and induction of this activity upon chronic ethanol consumption is reflected in increased rates of reduction of pNSP to pAP. The sharper pH-profile obtained with cytosol and NADPH as cofactor compared to NADH substantiates the evidence that NQOR activity is the predominant activity catalyzing cytosolic NADPH-dependent reduction of pNSP, as little competition with cytosolic ADH is observed when NADPH is the cofactor present.

While liver ADH was found not to be induced by the chronic ethanol feeding regimen used by us, the finding that ADH is the major NADH-dependent C-nitroso reductase activity towards pNSP in rat liver has important implications. The NADH/NAD⁺ ratio is dramatically increased upon oxidative *in vivo* metabolism of ethanol (Kalant and Khanna, 1969). It is therefore likely that while NADH is limiting in control animals, the increased cofactor availability upon ethanol ingestion would result in a potentiation effect due to induction of C-nitroso reductase activity coupled with the abundance of reduced cofactor. Ethanol ingestion is therefore likely to result in a shift

of the metabolite profile to more reduced species. These may be hydroquinones formed from quinones or hydroxylamino- and amino-derivatives formed from the respective nitroso compound. The partially reduced hydroxylamino- and amino compounds are necessary intermediates in the metabolism of nitroarenes and arylamines to the final DNA-binding electrophile. The increase in reductive metabolism of such intermediates upon ethanol ingestion therefore is likely to modulate the toxic and mutagenic sequelae associated with their bioactivation. This argument is strengthened by the observations of Dunn and Bernhard (1971) using purified horse liver ADH, who found that addition of ethanol in the presence of p-nitroso-N,N-dimethylaniline, NADH and ADH effects a shift in the equilibrium of the reaction towards the reduced nitroso compound. Thus, the presence of ethanol, as would be the case under conditions of ethanol ingestion, provides reducing equivalents for the reduction of the nitrosoaromatic by recycling NADH upon the oxidative metabolism of ethanol.

The present study also reveals that purified rat liver cytosolic NQOR catalyzes the NADH- and NADPH-dependent reduction of pNSP to p-aminophenol. The pH-optimum for the reaction found with the purified enzyme was about pH 6.5-6.8, which coincided with that of the cytosolic fractions. Below the pH optimum, NQOR-catalyzed NADPH-dependent C-nitrosoreductase activity was found to be approximately twice as efficient as the NADH-dependent activity. Purified NQOR has been reported to display two pH maxima in the reaction with menadione. The one in the more alkaline region was suggested to be due to the rapidly increasing rate of autooxidation of menadiol under more basic conditions, which decreases the concentration of product present and thereby prevents product inhibition of the enzyme. This is evidenced by the fact that addition of cytochrome c abolished the second pH maximum at alkaline pH (Maerki and Martius, 1960). The four-electron reduced product of pNSP, pAP probably does not

autooxidize rapidly, which is consistent with the single pH optimum observed as well as the rather high K_i towards pAP determined with a partially purified fraction from porcine heart (Horie and Ogura, 1980).

NQOR catalyzes the nitroreduction of 4-nitroquinoline-N-oxide (4-NQO) (DeFlora *et al.*, 1988) and displays stereoselectivity in the nitroreduction of dinitropyrenes (Hajos and Winston, 1991). Herein we have observed that highly purified NQOR catalyzes reduction of pNSP, but not of the parent nitrocompound p-nitrophenol, underlining the substrate specificity of this enzyme in the reduction of nitro- and C-nitrosoaromatics.

Ethanol ingestion is known to lead to cellular oxidative damage (Videla and Valenzuela, 1982) and, as a consequence, the induction of certain phase II enzymes such as glutathione-S-transferase. We observed that ethanol ingestion resulted in 70% and 20% increases over pair-fed controls in glutathione-S-transferase-catalyzed glutathione conjugation with respectively 1-chloro-2,4-dinitrobenzene (CDNB) and 4-NQO as substrates (see Chapter 2). The observed induction of NQOR parallels this response and may also be triggered by oxidative stress and/or its physiological consequences such as oxidative tissue damage, rather than by direct interaction of ethanol or its metabolites with regulatory elements. Recently, Nebert *et al.* (1990) put forth a model that describes the regulation of the *Nmo-1* gene, which encodes for NQOR and of other genes which encode for various phase II enzymes in a manner that is independent of the TCDD-receptor. This model is quite different from that by which, for example, 3-methylcholanthrene induces phase II enzymes, i.e. via direct interaction with the TCDD-receptor. The co-induction of NQOR and glutathione-S-transferase by ethanol ingestion seems to support this model, as ethanol ingestion induces a specific ethanol-inducible form of cytochrome P450 (CYP 2E1), but not the CYP 1A1 and CYP

1A2 isoforms, the induction of which is mediated through the TCDD-receptor. It is further noted that one of the genes induced in conjunction with *Gt-1* (glutathione-S-transferase) and *Nmo-1* (NQOR) is *Aldh-1*, which encodes for aldehyde dehydrogenase. We are currently exploring the effects of NQOR induction in cytosol on the mutagenicity of nitroarenes, arylamines and aromatic C-nitroso compounds.

5.6. Acknowledgments

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Chapter 6

Roles of rat liver cytosolic and microsomal fractions in the bioactivation of DNP: nitroreduction, metabolite formation and genotoxicity towards *S. typhimurium* TA1535/pSK1002

6.1. Abstract

Ethanol ingestion resulted in slight increases of cytosolic NAD(P)H:DNP nitroreductase activities. The enhanced rates of nitroreduction of DNP were, at least in part, due to induction of cytosolic NQOR as judged from the degree of inhibition of the reaction by dicoumarol. Cytosolic xanthine oxidase/xanthine dehydrogenase efficiently competed with NQOR in the reduction of DNP; up to 78% of the reaction was inhibited by allopurinol. All three dinitropyrene isomers were found to potently induce expression of the chimeric *umuC-lacZ* gene in *S. typhimurium* TA1535/pSK1002 in a dose-dependent manner. Addition of rat liver cytosolic fractions from ethanol- or pair-fed rats attenuated the induction of SOS-dependent bacterial DNA repair responses by 1,6- and 1,8-DNP. The 1,6-DNP isomer was more refractory to cytosolic deactivation than was the 1,8-DNP isomer. In contrast, 1,3-DNP was activated by cytosol above the direct bacterial response. The microsomal fractions from ethanol- and pair-fed rats were found to mediate effects opposite to that of cytosol with respect to isomer specificity.

While 1,3-DNP was potently deactivated by microsomes from either source, a deactivation response was observed with 1,6- and 1,8-DNP only at the higher concentrations of microsomal protein employed. The order of microsomal deactivation was found to be 1,3-DNP > 1,6-DNP > 1,8-DNP. Purified rat liver cytosolic NQOR slightly activated 1,6- and 1,8-DNP above the direct genotoxic response in the *umu*-test. No effect was seen towards the 1,3-DNP isomer. Despite the pronounced isomer specificity displayed by rat liver microsomal and cytosolic fractions, no effect of ethanol-ingestion on the genotoxicity of DNP towards *S. typhimurium* TA1535/pSK1002 was observed in any case. Furthermore, no significant differences in the rates of formation of metabolites from 1,6-DNP by either cytosolic or microsomal fractions were observed in HPLC-studies on metabolites formed from 1,6-DNP, consistent with the lack of effect of ethanol ingestion on the genotoxicity of DNP in the *umu*-test. The results emphasize the importance of stereoselective processes in the bioactivation of the DNP isomers. In addition, microsomal and cytosolic rat liver fractions evidently contain enzymes which apparently mediate opposing pathways of DNP activation. Thus, despite the remarkable potency of DNP to induce both, bacterial mutations in the Ames assay and DNA-repair responses as measured by the *umu* genotoxicity assay, very different molecular events appear to be in effect in the presence of rat liver subcellular fractions. These differences are discussed in light of the proposed pathways of DNP activation as well as in light of the mutational specificity of the three DNP isomers under study.

6.2. Introduction

Previously we have reported findings on the role of the cytosolic fraction in the mutagenic activation of DNP and aromatic amines and the differential effects observed upon pretreatment of rats with ethanol (Traynor *et al.*, 1988; 1991; Winston *et al.*, 1991). DNP were activated to a greater extent by cytosol from ethanol-fed rats than by that from pair-fed animals employing the Ames *Salmonella* tester strain TA100. The cytosolic activation displayed stereoselectivity and required the presence of NADPH. In turn, microsomes from ethanol-fed rats were found to be more active in detoxifying DNP than those from pair-fed control animals. More recently we have identified NQOR as a DNP nitroreductase which is induced in rats upon pretreatment with 3-methylcholanthrene or Aroclor-1254 (Hajos and Winston, 1991; see Chapter 3) as well as by ethanol ingestion (see Chapter 5).

The *umu*-genotoxicity assay has only very recently been used as an index for bacterial DNA-repair responses, which are thought to reflect the mutagenicity and carcinogenicity of test compounds in higher organisms. This assay has been employed mainly as a tool to evaluate and correlate the specific activities of P450 isoforms in different human microsomal samples with the mutagenic activation or deactivation of a variety of mutagens/carcinogens by these liver fractions (Shimada and Nakamura, 1987; Shimada *et al.*, 1989 a,b; 1990; Shimada and Guengrich, 1990). The basis of this assay has been extensively discussed (see section 1.5), however it cannot be overemphasized that the responsiveness of this assay reflects a bacterial DNA-repair response rather than the event of a particular mutation taking place at a specified site within the bacterial genome, as in the Ames assay. It should be noted that DNP were found to be only weakly mutagenic in strains TA1535 and 1537. In contrast, the respective daughter

strains TA98 and TA100 revealed the extraordinary mutagenic potency of all three DNP (Rosenkranz and Mermelstein, 1983). These strains contain the plasmid *pKM101*, which codes for certain DNA repair proteins (such as the *mucA* and *mucB* genes) and which are thought to mediate responses similar to the *umuD* and/or *umuC* gene products (Lambert *et al.*, 1989).

Shimada and Guengrich (1990) previously reported the effects of human and rat liver microsomal fractions on the genotoxicity of a series of direct acting mutagens, including 1,3-, 1,6- and 1,8-DNP in the *umu* genotoxicity test. These researchers observed that rat liver microsomes inhibited the genotoxicity of all three DNP isomers towards *S. typhimurium* TA1535/pSK1002 to similar extents, albeit the 1,3-DNP isomer appeared to be the one most potently deactivated. Studies employing pretreatment of the experimental animals with classical MFO inducers as well as antibodies raised against cytochrome P450 isoenzymes suggested that CYP 1A1 and CYP 1A2 are likely to be involved in the detoxification of 1,3-DNP. No clear correlation between the specific activity of any cytochrome P450 isoform and the detoxification of either 1,6- or 1,8-DNP by rat liver microsomes was observed. Purified rabbit liver NADPH-cytochrome P450 reductase was found to inhibit the genotoxicity of 1,6- and 1,8-, but not 1,3-DNP in the *umu*-test (Shimada and Guengrich, 1990). Furthermore, based on the lack of effect of anti-human CYP 2E1 antibody on the detoxification of DNP by human liver microsomes, these researchers concluded that the ethanol-inducible cytochrome P450 (CYP 2E1) is not involved in the microsomal detoxification of DNP.

The current section investigates the integrative contribution of cytosolic and microsomal fractions in the metabolism and mutagenic expression of DNP in the *umu*-test and attempts to evaluate the effects of ethanol ingestion on these processes. These

studies were complemented by metabolite analysis of 1,6-DNP by HPLC and the characterization of cytosolic NAD(P)H-dependent nitroreductase activities towards 1,3-, 1,6- and 1,8-DNP

6.3. Materials and Methods

6.3.1. Materials. Allopurinol, dicoumarol, ferricytochrome c, NADH, NADP⁺, NADPH, o-nitrophenyl- β -D-galactoside, Tris-base, Tris-Cl, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from Sigma (St. Louis, MO). [³H]-1,6-Dinitropyrene, 1,3-, 1,6- and 1,8-dinitropyrene were from Chemsyn Science Laboratories (Lenexa, KS) (>99% purity). Partially pure HPLC standards were kindly provided by Dr. F. A. Beland, Division of Biochemical Toxicology, National Center for Toxicological Research, Jefferson, AR, USA. Partially succinoylated ferricytochrome was prepared as described previously (see section 3.3).

6.3.2. Animals and Treatment. The ethanol-feeding protocol as well as that for the preparation of subcellular fraction has been described (Chapter 2). *Salmonella typhimurium* strain TA1535/pSK1002 (the "umu-strain") was a kind gift of Dr. Y. Oda, Osaka Prefectural Institute of Public Health, Osaka, Japan.

6.3.3. Methods.

6.3.3.1. Enzyme assays: Aerobic cytosolic DNP nitroreductase activities were measured as described (see section 3.4.). Rat liver cytosolic NQOR

(NAD(P)H:quinone oxidoreductase, E.C.1.6.99.2) was purified from rat liver cytosol by the method of Prochaska (1988) as described (see section 3.5) and NQOR activity was determined as previously reported (see section 3.5). Protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as a reference standard.

6.3.3.2. *umuDC* genotoxicity test: The *umu*-test was performed essentially as outlined by Oda *et al.* (1985) and Shimada and Nakamura (1987), but later modified to better suit the studies of nitroarenes, which are direct-acting bacterial mutagens (see footnote section 1.4), as indicated below (see also section 6.4.2.). A fresh culture was prepared by adding 100 μ l frozen permanent of *S. typhimurium* TA1535/pSK1002 to 5 ml Oxoid nutrient broth no. 2 containing 20 μ g/ml Ampicillin and incubated for 12-14 hours at 37°C overnight. To minimize competition between mammalian and bacterial metabolism, 400 μ l of the resulting overnight culture was diluted with 50 ml of fresh nutrient broth, which resulted in a bacterial cell density corresponding to an optical density of 0.025 to 0.05 units at 600 nm.

Cytosol or microsomes (50-250 μ g protein) were incubated in the presence of 0.02-0.16 μ M DNP and a NADPH-generating system. Final concentrations were 1 mM NADP⁺, 20 mM glucose-6-phosphate, 10 mM MgCl₂ and 125 mM potassium phosphate buffer pH 7.4 in a total volume of 0.25 ml. The reaction was initiated by adding the NADPH-generating system and allowed to proceed for 60 minutes at 37°C (Shimada and Guengrich, 1990). After this time, 750 μ l of the diluted, ice-cold overnight bacterial culture was added, which resulted in final toxicant concentrations of 0.005-0.04 μ M and it was incubated for another 2 hours at 37°C, unless otherwise indicated. Subsequently, tubes were placed on ice and 200 μ l aliquots removed for determination of β -galactosidase activity using the substrate *o*-nitrophenol- β -D-

galactoside (ONPG) according to the method of Miller (1971). The remainder of the bacterial solution was used to determine the bacterial cell density turbidometrically at 600 nm. Solvent (DMSO) control and positive control for mutagenicity¹ (5 μ M 4-NQO) were included in each experiment. Total units of β -galactosidase were calculated according to Miller (1971) using the Apple Macintosh Excel spreadsheet program. In all cases the background (DMSO) corrected rate is reported. Results are presented as the mean \pm SD of triplicate determinations unless otherwise indicated. Fifty percent growth inhibition as compared to buffer control was taken as a threshold for toxicity to the bacteria.

6.3.3.3. Metabolism of 1,6-DNP by cytosolic and microsomal fractions: Metabolism of 1,6-DNP was determined by reverse-phase HPLC following initial incubations of cytosolic or microsomal protein with [³H]-1,6-DNP. Dinitropyrene stock solutions were prepared in DMSO and stored under an Argon atmosphere at -80°C until use. For some microsomal incubations, the solvent (DMSO) was removed by liquid chromatography on a C-18 reverse phase disposable cartridge (Supelclean-C18, Supelco Inc., Bellefonte, PA). The sample (approximately 250 nmoles [³H]-1,6-DNP) was applied and the cartridge washed excessively with water to remove DMSO. Subsequently, the DNP was eluted with acetonitrile, evacuated under a stream of argon and resuspended in corn oil. Final incubations of 1 ml contained 0.2 mg cytosolic or microsomal protein, 20 μ M 1,6-DNP and 50 mM potassium phosphate buffer (KPi),

¹ The endpoint of the *umu*-test (i.e., bacterial DNA-repair induction as evidenced by the increased expression of the chimeric *umuC-lacZ* fusion protein) is very different from that of the Ames *Salmonella* mutagenicity assay (also see section 1.5). Because of the good correlation between positive responses in the *umu*-test and mutagenicities observed in the Ames test (Nakamura *et al.*, 1987) and for the sake of simplicity, positive responses in the *umu*-test in this report will sometimes be referred to as mutagenic response. This should by no means implicate any similarities of the molecular events involved in the complex processes leading to a positive response in either test system.

pH 7.4 (Djuric *et al.*, 1988). Reactions were started with the same NADPH-generating mixture employed in the *umu*-test, unless otherwise indicated. Following 15 min incubation at 37°C, 1,6-DNP metabolites and unmetabolized 1,6-DNP were extracted twice with 2 ml ethyl acetate and then twice with 2 ml chloroform:methanol (1:1), evaporated to dryness under argon and resuspended in 100 µl acetonitrile. 20 µl of this was injected onto a C18, 3 µm microorb column and analytes eluted with a linear gradient of 50% acetonitrile: 50% 0.1 M ammonium acetate buffer, pH 6.0 to 100 % acetonitrile (0.8 ml/min for 15 min beginning 15 min after injection). Thirty second fractions were collected into 5 ml of scintillation fluid and metabolites quantified on a Beckman LS6000 IC liquid scintillation counter. The metabolites 1-amino-6-nitropyrene and 1-nitroso-6-nitropyrene were tentatively identified by their expected elution pattern from a reverse phase column (Djuric *et al.*, 1985) as well as by the similar retention time of a synthetic 1-amino-8-nitropyrene standard. The 1-amino-8-nitropyrene standard was positively identified by direct-probe mass spectrometry after reverse-phase HPLC and comparison of the fragmentation patterns to that of commercially available 1-amino- and 1-nitropyrene.

6.4. Results

6.4.1. Cytosolic nitroreduction of DNP

DNP-nitroreductase activities in cytosol from ethanol- and pair-fed rats, determined with NADH or NADPH as the cofactors, are shown in Table 6.1. In all cases 1,6-DNP was reduced most efficiently, followed by the 1,8- and 1,3-DNP

isomers. This order of activity is consistent with previous reports (Djuric *et al.*, 1986, 1988, Hajos and Winston, 1991; see Chapter 3).

Table 6.1 Nitroreduction of dinitropyrenes by rat liver cytosol from ethanol- and pair-fed rats.

Compound:		1,3-DNP ^a	1,6-DNP	1,8-DNP
Treatment:	cofactor:	(nmol sCyt c reduced x min ⁻¹ x mg prot. ⁻¹)		
Pair-fed	NADH	0.4 ± 0.1 ^b	2.5 ± 0.1	1.4 ± 0.1
	NADPH	0.5 ± 0.1	0.9 ± 0.4	0.9 ± 0.2
Ethanol-fed	NADH	0.7 ± 0.1 (70%) ^c	2.5 ± 0.3 (0%)	2.0 ± 0.0 (40%)
	NADPH	0.6 ± 0.1 (30%)	2.5 ± 0.4 (180%)	1.5 ± 0.1 (70%)

^a The rate is expressed as nmol partially succinoylated ferricytochrome c reduced x min⁻¹ x mg prot.⁻¹

^b Results are mean ± SD of at least triplicate determinations with pooled cytosol from 6 rats within each treatment group.

^c Numbers in parentheses refer to the percent induction of nitroreductase activity as compared to pair-fed control.

6.4.1.1. NADPH-dependent activities: The DNP-nitroreductase activity of cytosol from ethanol-treated rats was increased 2.8-, 1.7- and 1.3-fold over pair-fed controls with 1,6-, 1,8- and 1,3-DNP as the substrate, respectively (Table 6.1). To further characterize the nature of this nitroreductase activity, incubations were performed in the presence of dicoumarol, a potent inhibitor of NQOR, and allopurinol, an inhibitor of the xanthine oxidase / xanthine dehydrogenase (XO/XD) couple. Both of these nitroreductase activities have been shown to be involved in the cytosolic reduction of 1-

NP and DNP (Howard and Beland, 1982; Djuric and McGunagle, 1990; Hajos and Winston, 1991). In control experiments with purified buttermilk xanthine oxidase it was found that 100 μ M dicoumarol inhibited the XO-catalyzed conversion of xanthine to urate by 50%. The per cent inhibition shown in Table 6.2 was therefore corrected by subtracting 50% of the allopurinol-sensitive rate from the portion of the rate inhibitable by 100 μ M dicoumarol. This corrective approach seemed further justified by the fact that the additive inhibition of dicoumarol and allopurinol in some cases was greater than 100 %, whereas in no case was complete inhibition observed when both inhibitors were present together in the incubation mixture (data not shown). Further data indicate that indeed dicoumarol is a potent inhibitor of XO which displays a K_i near the K_m for xanthine (see Appendix). NADPH-dependent nitroreduction of 1,6- and 1,8-DNP was inhibited approximately 50-60% by dicoumarol in cytosol from both ethanol- and pair-fed animals. Allopurinol inhibited the rate of reduction of these two isomers in cytosol from ethanol-fed rats by 30-40%, whereas only slight or no inhibition was seen in the pair-fed control system. In contrast, the dicoumarol-sensitive portion of 1,3-DNP nitroreduction was 65% in the ethanol- as compared to 22% in the pair-fed cytosolic system. Allopurinol was more effective as an inhibitor of 1,3-DNP nitroreduction in cytosol from pair-fed (60%) than from ethanol-fed (23%) rats. This preferential induction of 1,3-DNP nitroreduction by NQOR has been observed previously (Hajos and Winston, 1991, see Chapter 3) and coincides with the enhanced activation of 1,3-DNP by cytosol from ethanol-fed animals in the Ames test (Traynor *et al.*, 1988; Winston *et al.*, 1991) as compared to the other two DNP isomers under study.

Table 6.2 Nitroreduction of dinitropyrenes by rat liver cytosol from ethanol- and pair-fed rats: Effect of dicoumarol and allopurinol

a.) NADH-dependent activity:

Compound:		1,3-DNP ^a	1,6-DNP	1,8-DNP
Treatment:	Inhibitor:	(nmol sCyt c reduced x min ⁻¹ x mg prot. ⁻¹)		
Pair-fed	dicoumarol	0.4 ± 0.2 ^b (7%) ^c	0.9 ± 0.1 (21%)	0.3 ± 0.0 (26%)
	allopurinol	0.2 ± 0.1 (40%)	1.2 ± 0.2 (64%)	0.5 ± 0.1 (78%)
Ethanol	dicoumarol	0.3 ± 0.1 (33%)	0.8 ± 0.1 (22%)	0.5 ± 0.1 (37%)
	allopurinol	0.4 ± 0.1 (43%)	1.7 ± 0.0 (47%)	0.5 ± 0.0 (76%)

b.) NADPH-dependent activity:

Compound:		1,3-DNP ^a	1,6-DNP	1,8-DNP
Treatment:	Inhibitor:	(nmol sCyt c reduced x min ⁻¹ x mg prot. ⁻¹)		
Pair-fed	dicoumarol	0.2 ± 0.2 (22%) ^c	0.4 ± 0.0 (56%)	0.2 ± 0.0 (62%)
	allopurinol	0.2 ± 0.1 (60%)	1.2 ± 0.3 (0%)	0.7 ± 0.1 (22%)
Ethanol	dicoumarol	0.3 ± 0.07 (65%)	0.8 ± 0.1 (51%)	0.3 ± 0.1 (61%)
	allopurinol	0.4 ± 0.1 (23%)	1.7 ± 0.2 (32%)	0.9 ± 0.2 (39%)

^a The rate is expressed as nmol sCyt. c reduced x min⁻¹ x mg prot.⁻¹

^b The results are expressed as the mean + SD of at least triplicate determinations.

^c Numbers in parentheses are per cent inhibition of the rates of nitroreduction as compared to the absence of 100 μM dicoumarol or 10 μM allopurinol, respectively. The dicoumarol-sensitive part of the reaction was corrected for the observed partial inhibition of xanthine oxidase by dicoumarol. For further details, see text.

6.4.1.2. NADH-dependent activities: NADH-dependent nitroreduction of DNP was slightly elevated in cytosol from ethanol-fed rats with 1,3-DNP (1.7-fold) and 1,8-DNP (1.4-fold) as the substrate, whereas little difference was seen in nitroreductase activity towards 1,6-DNP. The ability of dicoumarol to inhibit cytosolic NADH-dependent nitroreduction of DNP in the ethanol- vs. the pair-fed system was pronounced with 1,3-DNP as the substrate (33% vs. 7%) whereas only slight differences were noted with 1,8-DNP (37% vs. 26%) and none with 1,6-DNP as the substrate. Allopurinol inhibited NADH-dependent DNP nitroreductase activities towards all three isomers in cytosol from ethanol- or pair-fed rats to about equal extent (Table 6.2), with the inhibitor being most effective with 1,8-DNP (76-78%) and least effective with 1,3-DNP (40-43%).

Dicoumarol was always more inhibitory with NADPH than with NADH as the cofactor. In other studies, NADPH-dependent C-nitrosoreductase activity was completely inhibited by dicoumarol whereas NADH-dependent reduction was essentially not (see Chapter 5). In turn, NADH-dependent DNP nitroreduction reactions were always inhibited to a greater extent by allopurinol than were NADPH-dependent reactions, in agreement with the reported cofactor specificity of XO / XD.

6.4.2. DNP-dependent induction of the *umu* gene in *S. typhimurium*

TA1535/pSK1002: Effect of rat liver subcellular fractions.

It has been shown previously that rat liver cytosol can augment the mutagenicity of DNP in the Ames test (Shah *et al.*, 1990, Traynor *et al.*, 1988) and that chronic ethanol consumption or pretreatment of animals with Aroclor-1254 (Traynor *et al.*, 1988, Shah *et al.*, 1990) can modulate this cytosolic activation of DNP. Cytosolic rat

liver NQOR is capable of activating all three DNP isomers to more potent mutagens in the Ames test (see Chapter 4). Furthermore, this cytosolic oxidoreductase activity is inducible upon chronic ethanol ingestion in rats (see Chapter 5). To further characterize alterations associated with chronic ethanol consumption, the *Salmonella umu* gene induction assay (Oda *et al.*, 1985, Shimada and Nakamura, 1987) was employed as an index for DNP-mediated bacterial genotoxicity and DNA-repair responses. Induction of the *umuDC* gene by DNP was studied as a function of chronic ethanol consumption using cytosolic and microsomal fractions as well as with purified rat liver cytosolic NQOR.

Initial studies employing the *umu* test were performed as a direct incubation assay according to the procedure by Shimada and Nakamura (1987), i.e. the toxicants and activating enzyme fractions were mixed directly with the bacteria and the reactions allowed to proceed for 2 hours. Experiments to evaluate the effects of cytosol on the genotoxicity of DNP in the *umu*-test, however indicated low sensitivity (data not shown), probably owing to the fact that the high concentration of bacteria outcompeted metabolism by the mammalian subcellular fractions. Therefore, similar to the preincubation protocol commonly employed in the Ames *Salmonella* mutagenicity test (Maron and Ames, 1983) and the preincubation procedure employed by Shimada and Guengrich (1990) in the *umu*-test, toxicants and enzyme fractions were preincubated for one hour before the addition of bacteria. To further optimize the assay procedure, the bacterial cell concentration was lowered by approximately ten-fold such that nitroreduction by mammalian cell fractions is likely to overcompensate that by bacterial enzymes. The revised preincubation procedure displayed much higher sensitivity to the addition of nonbacterial enzyme sources. The dose response to 1,3-, 1,6- and 1,8-DNP using this revised incubation protocol is shown in Figure 6.1.

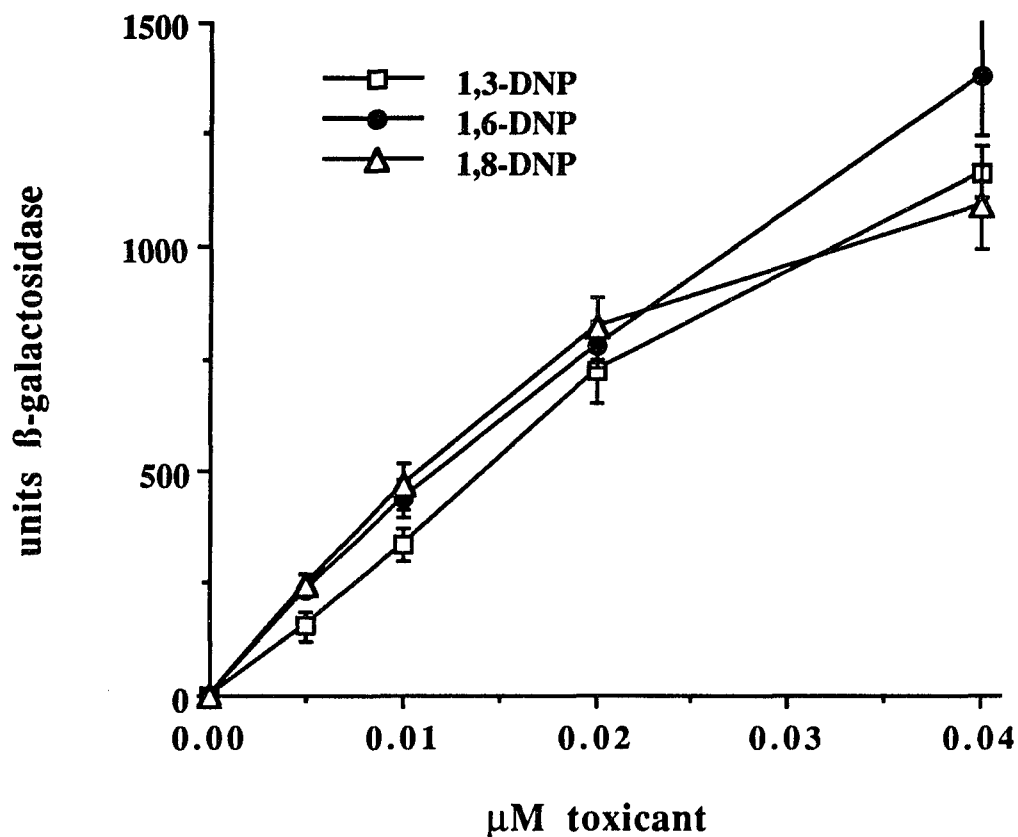


Figure 6.1. Dose response of the umu-test to 1,3-, 1,6- and 1,8-DNP. Increasing concentrations of DNP were incubated for two hours in the presence of *S. typhimurium* TA1535/pSK1002 and an NADPH-generating system. Results are means \pm SD of two independent experiments in triplicate. The background (DMSO) corrected rate is shown.

It probably should be pointed out however, that this revised procedure apparently is more sensitive to the cytotoxic effects imposed upon the bacteria by the test compounds, as evidenced by the growth inhibition that is observed at the very high

concentrations of toxicant employed. Whenever this was the case, the obtained results were either rejected or the more than 50% growth inhibition is indicated. The results reported herein only consider conditions under which a linear relationship between toxicant concentration and *umu* gene induction (in the absence of additional enzyme sources) was observed. This criterion for toxicity has been successfully employed in previous studies (T. Shimada, personal communication).

6.4.2.1. Effect of rat liver cytosol on umu-gene induction by DNP: The effect of increasing concentrations of cytosolic protein from ethanol- and pair-fed rats on the genotoxicity of 1,3-, 1,6- and 1,8-DNP in the *umu* assay was investigated. For these studies, the toxicant concentration was adjusted to final concentrations of 0.02 μ M DNP which resulted in β -galactosidase activities of 720 ± 68 , 779 ± 50 , and 818 ± 68 total units for 1,3-, 1,6- and 1,8-DNP, respectively (results are means \pm SD of two independent experiments in triplicate). These concentrations of toxicant allow either an activation or deactivation response to be seen. The cytosolic protein concentration was varied from 0-0.2 mg protein. Consistent with reports from our laboratory (Traynor *et al.*, 1988, Winston *et al.*, 1991), cytosol displayed pronounced isomer specificity (Figure 6.2). Cytosol from either source was found to negate 1,6- and 1,8-DNP-dependent induction of the *umu* gene over the range of protein concentrations studied. For equal protein concentration, 1,6-DNP was slightly more refractory to deactivation by cytosolic enzymes than was 1,8-DNP. In contrast, 1,3-DNP was found to be activated by cytosol at the lower protein concentrations employed, yet this activation reversed upon increasing the amount of protein present in the incubation mixture (Figure 6.2). No differences in either activation or deactivation of any DNP isomer between cytosol from ethanol-fed rats and that from pair-fed controls were noted.

Subsequent studies evaluated the effect of toxicant concentration on the cytosol-mediated modulation of DNP mutagenicity in the *umu*-test. For these studies, a cytosolic protein concentration of 0.1 mg was employed, a concentration which was found to display the most marked effects in the previous experiments, and the concentration of DNP was varied in the range of 0.005-0.04 μ M (Table 6.3). This concentration range spans from marginal induction of the *umu* gene by DNP (i.e., approximately twice solvent background) to very high levels of induction (Shimada *et al.*, 1988).

Cytosol from ethanol- and pair-fed rats potentially increased the mutagenicity of 1,3-DNP over the range of toxicant concentration studied (Table 6.3). In contrast, 1,6- and 1,8-DNP were detoxified by cytosolic enzymes to metabolites which were apparently less potent in inducing *umu*-dependent DNA-repair responses in *S. typhimurium* TA1535/pSK1002. Cytosol from either source was found to be more active in detoxifying 1,8-DNP than it was with 1,6-DNP. At the highest concentrations of toxicant tested, virtually no detoxification of either 1,6- or 1,8-DNP was observed, consistent with previous reports on the S9-mediated detoxification of these two DNP isomers in TA100 employing the Ames test (Traynor, 1988). As before, no significant differences between the ethanol- and pair-fed cytosolic systems were observed with any of the DNP isomers under the experimental conditions employed.

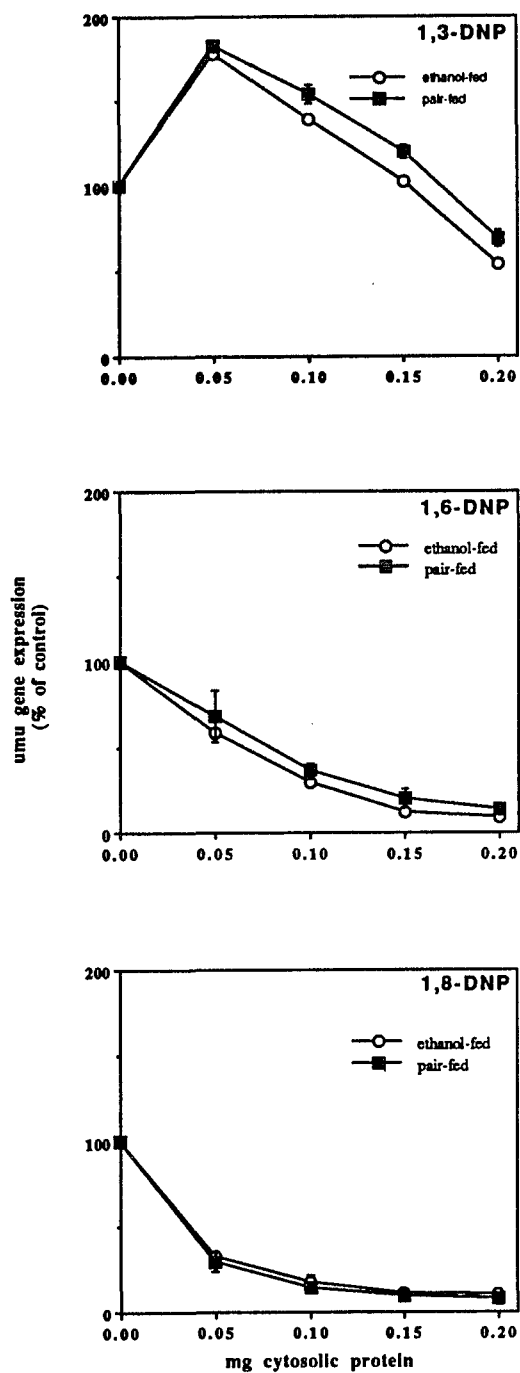


Figure 6.2. Activation and inactivation of 1,3-, 1,6- and 1,8-DNP by liver cytosol from ethanol and pair-fed rats. The indicated amounts of cytosolic protein from 6 animals within each treatment group was incubated in the presence of $0.02 \mu\text{M}$ DNP and the activity of (in)activation was determined in the presence of an NADPH-generating system. The control (100%) values of 1,3-, 1,6- and 1,8-DNP were 409 ± 15 , 745 ± 10 and 795 ± 1 total units β -galactosidase, respectively.

Table 6.3. Effect of ethanol- and pair-fed cytosol on the genotoxicity of DNP in the *umu*-test: Toxicant response

Treatment concentration		1,3-DNP	1,6-DNP total units β -galactosidase	1,8-DNP
no cytosol	0.005 μ M	155 \pm 33 ^a	233 \pm 2	248 \pm 26
	0.010 μ M	339 \pm 35	437 \pm 42	468 \pm 51
	0.020 μ M	720 \pm 68	779 \pm 50	818 \pm 68
	0.040 μ M	1165 \pm 55	1382 \pm 137	1089 \pm 94
Pair-fed	0.005 μ M	313 \pm 34 ^b	13 \pm 20	14 \pm 8
	0.010 μ M	604 \pm 62	57 \pm 6	31 \pm 8
	0.020 μ M	1215 \pm 148	306 \pm 2	103 \pm 22
	0.040 μ M	1536 \pm 10	1099 \pm 122	640 \pm 82
Ethanol-fed	0.005 μ M	256 \pm 21	4 \pm 4	24 \pm 13
	0.010 μ M	524 \pm 29	57 \pm 18	50 \pm 34
	0.020 μ M	982 \pm 72	344 \pm 47	190 \pm 114
	0.040 μ M	1382 \pm 137	1095 \pm 124	759 \pm 294

^a Results are mean \pm SD of two independent experiments in triplicate. The background (DMSO) activity was subtracted.

^b Pooled cytosol from 6 animals of each treatment group was used at 0.1 mg protein.

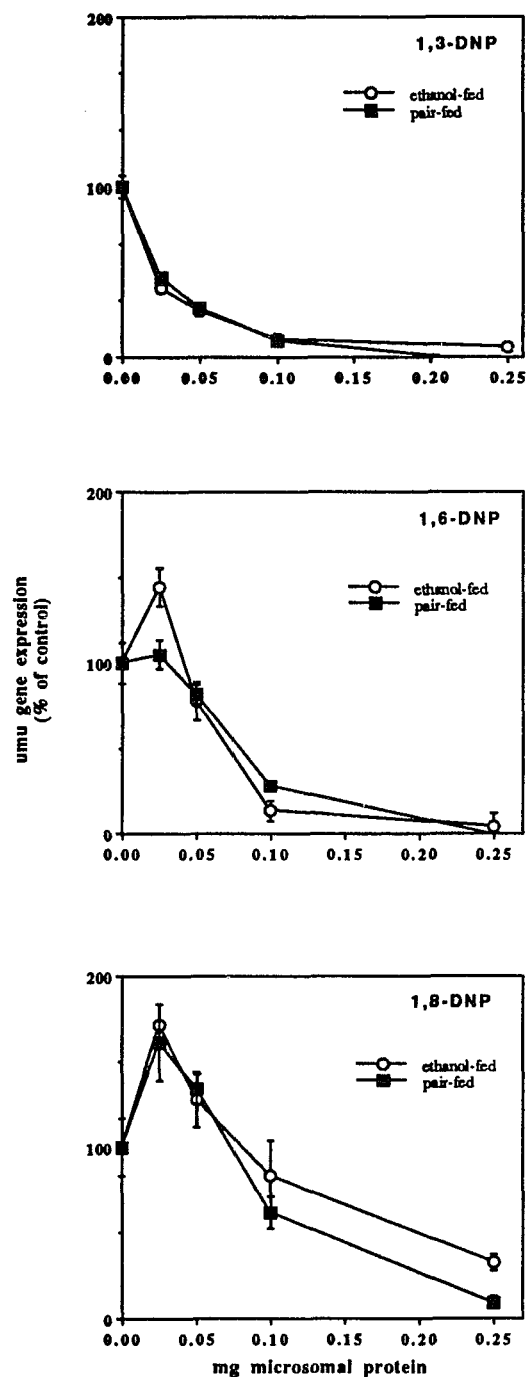


Figure 6.3. Activation and inactivation of 1,3-, 1,6- and 1,8-DNP by liver microsomes from ethanol and pair-fed rats. The indicated amounts of microsomal protein from 6 animals within each treatment group was incubated in the presence of $0.02 \mu\text{M}$ DNP and the activity of (in)activation was determined in the presence of an NADPH-generating system. The control (100%) values of 1,3-, 1,6- and 1,8-DNP were 489 ± 31 , 1015 ± 120 and 976 ± 161 total units β -galactosidase, respectively.

6.4.2.2. Effect of rat liver microsomes on *umu*-gene induction by DNP. The effect of microsomes on the mutagenicity of 1,3-, 1,6- and 1,8-DNP was studied with microsomes from ethanol- and pair-fed rats. The response of *S. typhimurium* TA1535/pSK1002 to the presence of 0.02 μ M DNP is shown in Figure 6.3 as a function of increasing concentrations of microsomal protein. Interestingly, while cytosol augmented the mutagenicity of 1,3-DNP in the *umu*-test, but attenuated that of 1,6- and 1,8-DNP, the reverse was true when microsomes were employed as the exogenous enzyme source. Thus, the 1,3-DNP isomer was potently detoxified by microsomes from both, ethanol- and pair-fed rats. Microsomal protein concentrations as low as 0.1 mg/ml virtually abolished *umu*-gene induction by 1,3-DNP. On the contrary, 1,6- and 1,8-DNP were activated (to approximately 50% and 80%, respectively) above the direct-acting mutagenicity at low concentrations of microsomal protein (i.e., 0.025 mg/ml). At higher protein concentrations, a detoxification response was seen. For equal protein concentrations, 1,8-DNP was more potently activated at the lower microsomal protein concentrations and less potently deactivated in the presence of higher concentrations of microsomal protein, than the 1,6-DNP isomer (Figure 6.3).

6.4.2.3. Effect of purified rat liver NQOR on *umu*-gene induction by DNP. The effect of increasing concentrations of purified NQOR on the mutagenicity of DNP is shown in Figure 6.4. Lower concentrations of NQOR slightly increased *umu*-gene induction by 1,6- and 1,8-DNP, as compared to the absence of the enzyme. This activation response was reversed when more than 100 units of NQOR was present in the incubation mixture. At approximately 75 units of NQOR, expression of β -galactosidase activity by 1,6- and 1,8-DNP increased from 420 and 450 units β -galactosidase to approximately

580 and 620 units β -galactosidase, respectively. Addition of NQOR showed virtually no effect on the mutagenicity of 1,3-DNP at any concentration of enzyme tested.

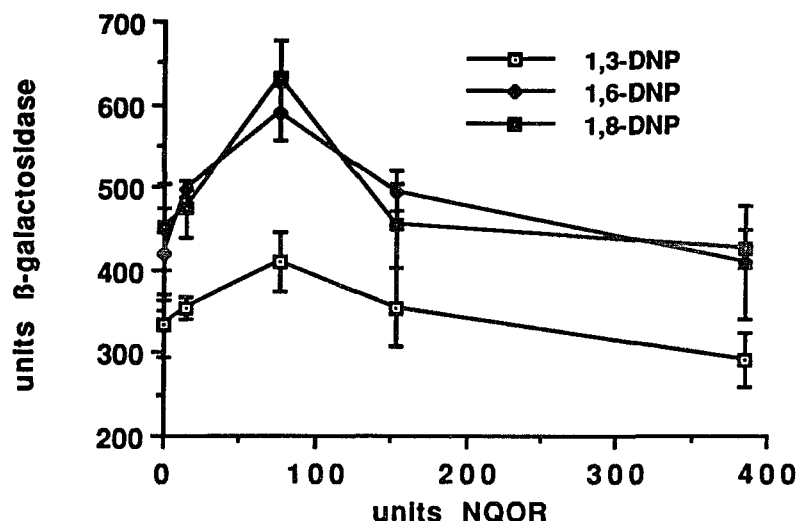


Figure 6.4. Effect of purified NQOR on *umu*-gene induction by 1,3-, 1,6- and 1,8-DNP. Increasing amounts of NQOR were incubated in the presence of 0.02 μ M DNP and an NADPH-generating system. The background (DMSO) rate of β -galactosidase activity has been subtracted.

To further validate these results, the toxicant concentration was varied over the range of 0.005-0.02 μ M DNP while holding the concentration of NQOR constant at 75 units. Under these conditions, purified NQOR displayed only marginal effects on the mutagenicity of the DNP isomers in the present test system (data not shown). Increasing the incubation period in presence of bacteria, toxicant and purified enzyme from 2 hours to 4 hours however, was revealing. At the concentration of NQOR employed, the mutagenicity of 1,8-DNP was almost doubled over the range of toxicant concentration studied (Figure 6.5). This was paralleled by significant increases in the

umu-gene response to concentrations of 1,6-DNP above 0.005 μ M in the presence of 75 units NQOR. In contrast, NQOR had no effect on the mutagenicity of 1,3-DNP at any concentration of toxicant, consistent with the data presented in Figure 6.4.

6.4.3. Formation of 1,6-DNP metabolites in rat liver cytosolic and microsomal fractions: Effect of ethanol-ingestion

The previous sections have shown that ethanol-ingestion leads to a slight elevation of cytosolic DNP nitroreductase activities. Enhanced rates of DNP nitroreduction can be, in part, be ascribed to induction of cytosolic NQOR. Furthermore, the induction of CYP 2E1 in microsomes from ethanol-fed rats has been documented (see Chapter 2). Despite the apparent increases in activity of these two enzymes, both of which have been invoked to play a role in the metabolism of DNP (Traynor *et al.*, 1988, Shimada and Guengrich, 1990, Hajos and Winston, 1991), no effect of ethanol-ingestion on the mutagenicity of DNP was observed employing the *umu*-test. To address these discrepancies, the metabolism of 1,6-DNP by rat liver cytosolic and microsomal fractions was studied by HPLC using radiolabelled substrate. This isomer was chosen because it is reportedly a better substrate for nitroreductases (including NQOR) than are 1,3- and 1,8-DNP and furthermore is a far more potent mutagen than 1,3-DNP.

The previous chapters (see Chapters 1, 2 and 5) have emphasized the importance of ethanol-mediated changes in the ratio of oxidized vs. reduced pyridine nucleotides. In order to illustrate this point, the time-dependent formation of metabolites from 1,6-DNP by cytosol from ethanol-fed rats was studied in the presence of NADH.

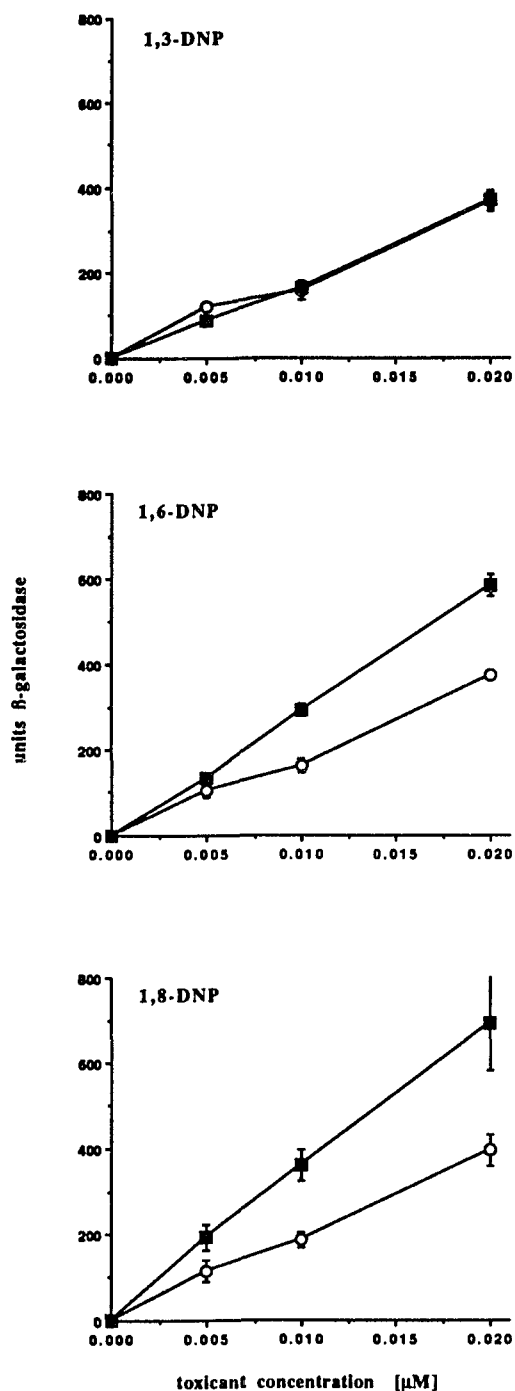


Figure 6.5 Activation of 1,3-, 1,6- and 1,8-DNP by purified rat liver NQOR. The indicated concentrations of DNP were incubated in the absence (open symbols) or presence (closed symbols) of 75 units NQOR and the activity of activation was determined in the presence of an NADPH-generating system after one hour of preincubation and four hours of incubation in the presence of *S. typhimurium* TA1535/pSK1002. Results are means \pm SD of triplicate determinations. The background (DMSO) corrected activity is shown.

Incubation of cytosol from ethanol-fed rats in presence of NADH led to a time-dependent increase in the formation of the corresponding partially reduced metabolites of 1,6-DNP (Figure 6.6). Assignment of the probable structures of the metabolites formed is in accordance with previous reports (Djuric *et al.*, 1985; 1986; 1988; Heflich *et al.*, 1987) however, it is realized that a more solid structural identification would be necessary to positively identify these metabolites. The rate of formation of 1-nitroso-6-nitropyrene peaked at 30 minutes and decreased afterwards, while formation of the corresponding amine increased up to 60 minutes, consistent with a sequential path of amine formation via the nitroso-intermediate. In order to be able to quantify both of these cytosolic metabolites, all subsequent incubations were performed for 15 minutes.

The NADPH- and NADH-dependent rates of formation of metabolites of 1,6-DNP in rat liver cytosolic incubations are shown in Tables 6.4 A and B, respectively. The retention time for 1,6-DNP under the chromatographic conditions employed was 25 minutes, that of the tentatively identified metabolites 1-amino-6-nitropyrene and 1-nitroso-6-nitropyrene 14 minutes and 27 minutes, respectively. Compared to the NADPH-dependent rates, NADH-dependent rates of metabolite formation appear to be lower in rat liver cytosolic fractions from ethanol- and pair-fed rats (Table 6.4). This was considerably more pronounced in the rates of formation of 1-nitroso-6-nitropyrene than in that of 1-amino-6-nitropyrene. The lower efficiency of NADH to serve as a cofactor for cytosolic reductases has been observed previously (Djuric *et al.*, 1986; Heflich *et al.*, 1986). No significant differences between the ethanol-fed and pair-fed cytosolic systems were noted with respect to the rate of formation of either one of these cytosolic metabolites. The data presented support the previous observations that various nitro- and nitroso-reductases are present in rat liver cytosol which display cofactor- as well as substrate specificity (also see Chapters 3-5).

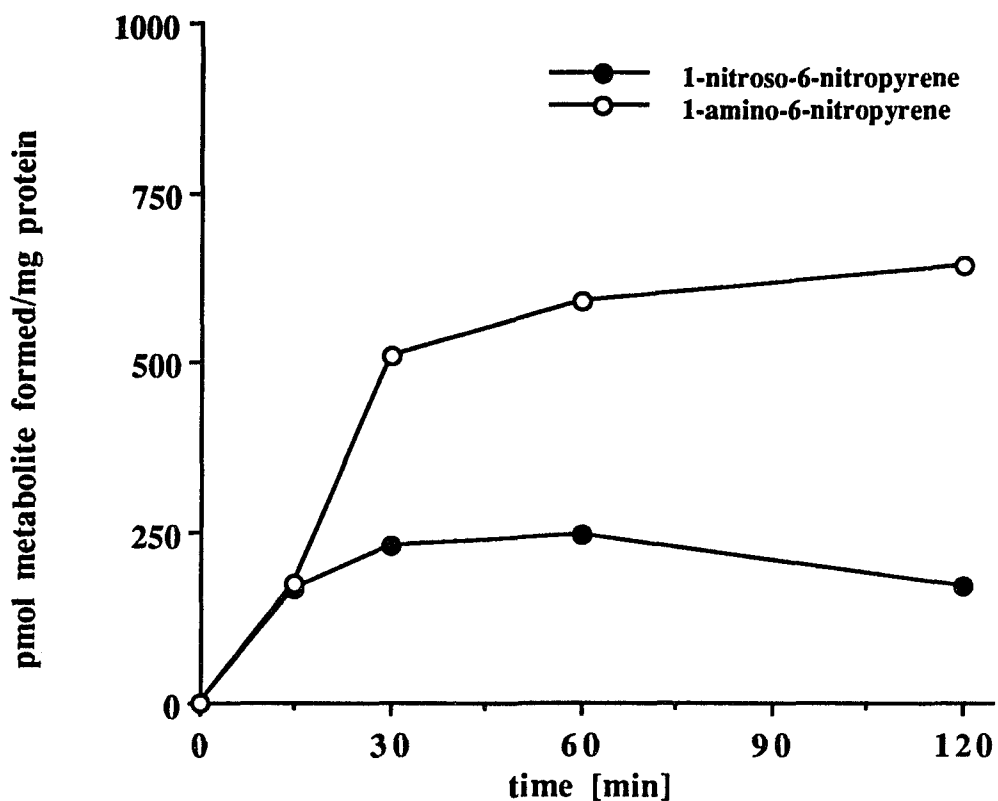


Figure 6.6 NADH-dependent metabolite formation from 1,6-DNP by rat liver cytosol from ethanol-fed rats: Dependence on time. Rat liver cytosol from ethanol fed rats (0.2 mg) was incubated in the presence of 1 mM NADH and 20 μ M [3 H]-1,6-DNP for the indicated periods of time. Subsequently the reaction was terminated and the metabolites analyzed as described in Materials and Methods. The metabolites eluting at 15 min and 27 min have been tentatively identified as 1-amino-6-nitropyrene and 1-nitroso-6-nitropyrene, respectively.

Table 6.4 NAD(P)H-dependent 1,6-DNP metabolism by rat liver cytosol: Effect of pretreatment.

A. NADPH-dependent activities.

retention time:		amine (15 min)	nitroso (27 min)	metabolite A (29 min)	metabolite B (31 min)
Pretreatment:	cofactor	pmol metabolite formed/15 min			
Pair-fed	NADPH	400 ± 5 ^a	700 ± 7	340 ± 16	470 ± 163
Ethanol-fed	NADPH	360 ± 87	750 ± 17	470 ± 61	440 ± 56

^a Results are presented as means ± SEM of duplicate determinations with pooled fractions from 6 animals in each treatment group.

B. NADH-dependent activities.

		amine	nitroso	metabolite A	metabolite B
Pretreatment:	cofactor	pmol metabolite formed/15 min			
Pair-fed	NADH	220 ^b	160	300	380
Ethanol-fed	NADH	220	140	310	380

^b Results are from a single determination with pooled fractions from 6 animals in each treatment group.

Incubation of cytosolic fractions from both, ethanol- and pair-fed rats with 1,6-DNP resulted in the formation of at least two further metabolites which eluted at approximately 29 min and 31 min under the chromatographic conditions employed (Table 6.4). These metabolites are therefore likely to be even less polar than the parent nitro compound 1,6-DNP. The nature of these metabolites has not been identified; it is noteworthy however, that cytosolic incubations employing fractions derived from ethanol-fed rats resulted in greater rates of formation of these relatively nonpolar metabolites than those derived from pair-fed controls (Table 6.4). In this regard it should perhaps be mentioned, that incubation of 1-NP with purified xanthine oxidase has been found to result in the formation of at least one metabolite of 1-NP which eluted after the parent 1-NP under chromatographic conditions similar to those employed by us (Bauer and Howard, 1990). This metabolite was suggested to be 1,1'-azo- or 1,1'-azoxypyrene, but has not been positively identified.

Figure 6.7 shows the radioactivity profile obtained from incubation of 1,6-DNP with rat liver cytosolic NQOR (1000 units) in the presence of an NADPH-generating system. While it is realized that the rate of metabolite formation appears to be rather low, the profile obtained is virtually identical with that employing rat liver cytosolic fractions as the catalyst. The tentatively identified metabolites 1-nitroso- and 1-amino-6-nitropyrene were both detected (Figure 6.7). Formation of the amine by NQOR is consistent with our previous observations that rat liver cytosolic NQOR possesses nitro- as well as nitrosoreductase activity (Chapters 3-5).

Incubations of 1-NP with rat liver microsomal fractions leads to the formation of a variety of ring hydroxylated metabolites as well as 1-aminopyrene. In contrast, no ring hydroxylated derivatives of DNP have been detected to date (Djuric *et al.*, 1988; Beland and Kadlubar, 1990). Furthermore, the problem of solvent inhibition of

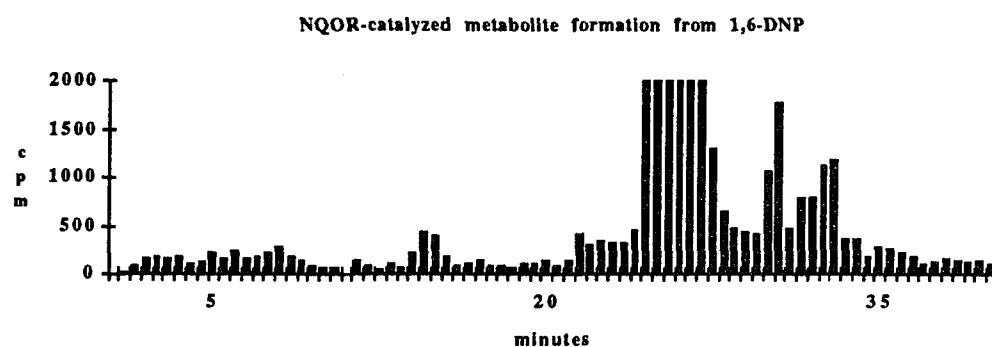


Figure 6.7 Metabolite formation from 1,6-DNP by purified rat liver NQOR. Purified rat liver NQOR (1000 U) was incubated in the presence of 20 μ M [3 H]-1,6-DNP and an NADPH-generating system for 15 minutes. Subsequently the reaction was terminated and the metabolites analyzed as described in Materials and Methods. The metabolites eluting at 15 min and 27 min have been tentatively identified as 1-amino-6-nitropyrene and 1-nitroso-6-nitropyrene, respectively.

CYP 2E1 has been discussed (Chapter 2; Shimada *et al.*, 1989). In order to circumvent this problem and to test for a role of CYP 2E1 in the metabolism of 1,6-DNP, we have performed rat liver microsomal incubations in the presence of 1,6-DNP, which was delivered in corn oil. Extra care was taken to remove any residual DMSO (see Materials and Methods for details). In these incubations, the concentration of 1,6-DNP was increased 5-fold to result in final concentrations of approximately 100 μ M 1,6-DNP. In order to compensate for the effects of corn oil, which will counteract the partitioning into the microsomal lipid phase, the 1,6-DNP concentration was increased. The highly hydrophobic DNP are expected to partition into the microsomal lipid. This has been pointed out for the case of microsomal incubations containing 1-NP, where low micromolar concentration of 1-NP result in local concentrations of about 100 mM within the microsomal lipid bilayer (Howard and Purvis, 1989).

The results obtained following this experimental protocol are shown in Figure 6.8 following incubations with microsomes from ethanol- and pair-fed rats. The majority of the radioactivity coeluted with 1,6-DNP. The only metabolites detectable were those previously observed in incubations using rat liver cytosol as the enzyme source. Unfortunately, the presented data do not allow one to rigorously exclude an effect of ethanol ingestion on the microsomal metabolism of 1,6-DNP, nor do they allow one to imply or rule out a role of CYP 2E1 in the metabolism of this compound. It is apparent however, that if ring-hydroxylated metabolites were formed in the incubation mixture, these are not likely to be major metabolites.

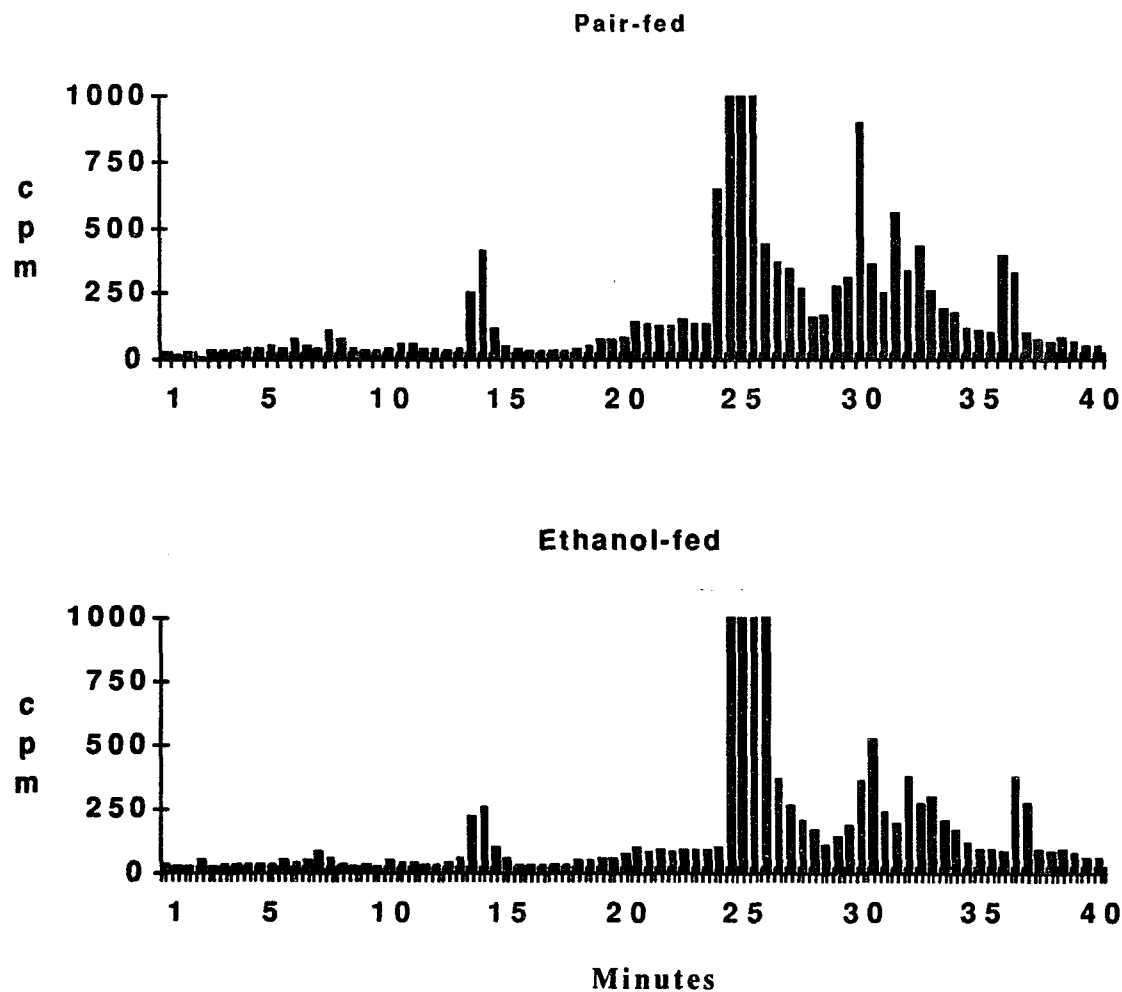


Figure 6.8. Elution profile of 1,6-DNP by reverse-phase HPLC after incubation with rat liver microsomes. Pooled microsomes (0.2 mg protein) from ethanol- and pair-fed rats were incubated in the presence of $\sim 100 \mu\text{M}$ [^3H]-1,6-DNP and an NADPH-generating system for 15 minutes. The reaction was terminated and the metabolites analyzed as described in Materials and Methods. Results are presented as cpm/ 15 minutes/ mg microsomal protein from a single determination with each treatment group.

6.5. Discussion

Previous studies (Traynor *et al.*, 1988; Shah *et al.*, 1990; Wilcox *et al.*, 1982; Djuric *et al.*, 1985; 1986; 1988) have shown that both microsomal and cytosolic enzyme activities are involved in the metabolism of DNP. The first step in the activation of these compounds involves nitroreduction to the partially reduced metabolites, namely the nitroso- or hydroxylamino compounds, which are capable of interacting with cellular macromolecules such as DNA. Previously our laboratory reported on the cytosolic activation of DNP in the Ames mutagenicity assay, employing *S. typhimurium* TA100 (Traynor *et al.*, 1988) and showed that cytosol from ethanol-fed animals was more efficient at enhancing the mutagenicity of DNP than that from pair-fed controls. This effect was most pronounced with 1,3-DNP, less with 1,6-DNP and absent with 1,8-DNP.

To better understand the role of cytosol in the metabolism of DNP and the differential effects observed upon ethanol ingestion, cytosolic nitroreductase activities were investigated. The potent induction of cytosolic NQOR, in concomitance with cytosolic NAD(P)H-DNP nitroreductase induction in animals pretreated with Aroclor-1254 has been discussed in Chapter 3. In contrast, cytosolic NAD(P)H-DNP nitroreductase activities were found to be elevated only slightly in preparations from ethanol-fed rats over pair-fed controls. The increase in DNP nitroreduction was expressed to a greater extent in NADPH- than in NADH-linked activities, possibly reflecting competition with other cytosolic nitroreductases. In this regard, alteration of cytosolic xanthine oxidase/xanthine dehydrogenase activities upon prolonged exposure to ethanol deserves further consideration (Abbondanza *et al.*, 1989). Purified XO has been shown to catalyze the reduction of 1-nitropyrene and 1,6-dinitropyrene to DNA-

binding species (Howard and Beland, 1982; Djuric and McGunagle, 1990). The preferential induction of NADPH-dependent nitroreductase activities might thus be explained by the cofactor specificity of the XO / XD couple, resulting in a lower efficacy of NQOR as a DNP nitroreductase due to competition with other activities. This is suggested by the allopurinol inhibition studies in which it was found that NADH-dependent DNP nitroreduction was generally more sensitive to the inhibition by allopurinol than were NADPH-linked activities. Alternatively, the induction of other NQOR isoenzymes might be considered, which have been reported to be present in mouse, rat and human liver cytosol, and whose cofactor and substrate specificity has not been well characterized (see discussion in chapter 3).

We have employed the *umu* genotoxicity assay to study DNP-mediated bacterial repair responses and their alteration upon addition of rat liver subcellular fractions. The *umu*-test, which is based on the response of the *umuDC* gene to toxicants which induce bacterial DNA-repair responses, revealed the potent mutagenicity of all three DNP isomers. This is consistent with previous reports in the Ames *Salmonella* mutagenicity test (Rosenkranz and Mermelstein, 1983; 1985; Maeda *et al.*, 1986; Fifer *et al.*, 1988). Addition of rat liver cytosolic fractions to incubations containing 1,6- or 1,8-DNP and *Salmonella typhimurium* TA1535/pSK1002 resulted in a potent deactivation of these chemical mutagens. In contrast, the 1,3-DNP isomer displayed a biphasic kind of behavior; activation was seen at lower concentrations of cytosolic protein while a detoxifying response resulted when the amount of exogenously added protein was increased. Previous studies from our laboratory parallel these observations in that 1,3-DNP was activated in the Ames test by cytosol, whereas only slight effects were noted with 1,6-DNP and none with 1,8-DNP, at a fixed concentration of cytosolic protein using TA100. Microsomal fractions appeared to mediate somewhat opposite effects

than cytosol. Microsomes from ethanol- or pair-fed rats potently deactivated 1,3-DNP in the *umu*-test. In contrast, 1,6-DNP was detoxified only at the higher protein concentrations employed and 1,8-DNP was most refractory to microsome-mediated detoxification. Thus, despite the apparent differences between the *umu*-test and the Ames assay, the same relative tendencies of the DNP isomers towards microsomal or cytosolic activation/deactivation steps are observed. It should be pointed out however, that no effect of ethanol ingestion on either cytosolic or microsomal metabolism of DNP was observed in the *umu*-test. Results employing the Ames test indicated rather pronounced effects of ethanol ingestion with either microsomes or cytosol as the enzyme source, depending on the DNP isomer under consideration. These differences are not completely understood, but possible causes will be suggested below.

That microsomes mediate effects opposite to that of cytosol is not without precedence. An example is from studies by Wilcox *et al.* (1982), who employed a yeast system to investigate the effect of rat liver fractions on the mutagenicity of 1,6-DNP towards this organism. In a preceding report, remarkably entitled "The genetic activity of dinitropyrenes in yeast: unusual dose response curves for induced mitotic gene conversion" (Wilcox and Parry, 1981), it was found that the toxicity and genetic activity of 1,6- and 1,8-DNP decreased at the higher toxicant concentrations employed. Subsequently these researchers observed that rat liver S9 and cytosolic fractions were activating 1,6- and 1,8-DNP, while the corresponding microsomal fractions decreased the mutagenicity towards *S. cerevisiae* of the DNP under study. It was further noted that apparently "two conflicting enzyme systems for DNP may be operating, i.e. a cytosolic system which enhances the mutagenic activity of DNP, and a microsomal system which reduces activity". Within a S9 mixture, the cytosolic effects were found to overshadow those of the microsomal fraction. That microsomal fractions deactivate and cytosolic

fractions activate DNP is also the case in the Ames *Salmonella* mutagenicity test (Traynor *et al.*, 1988; Winston *et al.*, 1991).

Shimada and Guengrich (1990) have reported that human and rat liver microsomes deactivate all three DNP in the *umu*-test. With human microsomes, the 1,3-DNP isomer was found to be most actively detoxified, followed by 1,6-DNP, while 1,8-DNP was most refractory to microsomal inactivation, which is consistent with our observations on rat liver microsomes. In comparison to our results, it appears that the slight differences observed with 1,6- and 1,8-DNP may be due to the different experimental protocols employed. While these researchers apparently chose microsomal protein concentrations similar to those employed in our studies, the reaction was terminated by heat-inactivation of the liver enzyme fractions after one hour of preincubation before assessing the genotoxicity towards *S. typhimurium* TA1535/pSK1002. Unfortunately, Shimada and Guengrich do not report on the effect of increasing rat liver microsomal protein concentrations on the genotoxicity of DNP in the *umu*-test, so that a direct comparison of their results to those obtained by us is difficult. It is possible that heat-inactivation may destroy some of the microsomally formed, reactive metabolites of DNP.

Incubations of 1-NP in the presence of microsomes leads to the formation of a variety of ring hydroxylated products, the rate of formation or isomer distribution of which does not seem to be affected dramatically upon ethanol ingestion (Howard *et al.*, 1987 b; 1988). The data presented on the metabolism of 1,6-DNP by microsomal fractions from ethanol- or pair-fed rats are consistent with previous reports on the lack of oxidative microsomal metabolism of DNP (Djuric *et al.*, 1988; Howard and Purvis, 1989; Beland and Kadlubar, 1990).

Our results in the *umu*-test underscore the importance of isomer specificities in the metabolism of DNP. Notably, while 1,3-DNP is the isomer reduced the slowest by rat liver cytosolic nitroreductases, it is also the isomer which is most strongly activated by cytosolic fractions in the Ames test (Traynor *et al.*, 1988, Traynor 1988, Winston *et al.*, 1991) and the only one which is activated by cytosolic fractions in the *umu*-test. We have further observed that purified rat liver NQOR increased the mutagenicity of 1,6- and 1,8-DNP, but not 1,3-DNP in the *umu*-test. Contrary to the observations in the *umu*-test, all three DNP were potently activated by purified NQOR in the Ames test using the tester strain TA98 (Chapter 4). Interestingly, the lack of activation of 1,3-DNP by NQOR in the *umu*-test is paralleled by the lack of deactivation in the presence of purified NADPH-P450 reductase in the same test system, as observed by Shimada and Guengrich (1990). 1,3-DNP is also the only DNP isomer which shows an attenuated mutagenic response in the nitroreductase-deficient strain TA98 NR (Rosenkranz and Mermelstein, 1983).

The activation of DNP is thought to proceed via nitroreduction and subsequent acetylation. It has been suggested, that peri-substituted nitroarenes are not good substrates for these acetylases (Djuric *et al.*, 1985). The results observed may therefore be a reflection of the relative rates of nitroreduction (by NQOR and bacterial nitroreductases) versus acetylation by the bacterial acetylases. Thus, 1,6- and 1,8-DNP, the better substrates for NQOR are effectively reduced by the enzyme and subsequently acetylated by the bacterial acetylases. If nitroreduction is the rate limiting step in the mutagenic expression of these two isomers, addition of NQOR to bacterial incubations will lead to an increase in the mutagenicity, as was observed. In the case of 1,3-DNP (the only DNP isomer with two nitrogroups in peri-position), acetylation may be limiting and thus addition of NQOR would be without any effect. A similar mechanism

may hold true for NADPH-cytochrome P450 reductase, albeit different products may result from incubations of DNP with this reductase. Interestingly, Shimada and Guengrich noted this possibility by stating that "it is not clear why nitroreduction is a detoxification process under some conditions but not under others". The situation of possibly competing pathways becomes exceedingly complex in the case of microsomal or cytosolic incubations, as microsomes contain nitroreductase(s) and deacetylase(s) and cytosol catalyzes nitro- and nitroso-reduction as well as acetylation steps. The differences observed between the *umu*-test and the Ames test (Traynor, 1988) may be due, at least in part, to differences in the ratio of bacterial to mammalian enzymes present. Furthermore, the relative involvement of the various nitroreductases that catalyze nitroreduction and secondary reductive metabolism of partially reduced nitroaromatics i.e., nitroso- and hydroxylamino intermediates is of critical importance in the mutagenic expression of nitroarenes. In this regard, some C-nitroso compounds are readily reduced by alcohol dehydrogenase (ADH) whereas the parent nitrocompound is not. We have previously alluded to the dramatic increase in the NADH/NAD⁺ ratio upon chronic ethanol ingestion and the catalytic activities of ADH as well as NQOR (see Chapter 5). While the involvement of ADH in the metabolism and mutagenicity of nitro-/nitrosoaromatics was not investigated in the current section, studies are underway in our laboratory to explore the role of ADH as a function of ethanol ingestion in the *in vitro* and *in vivo* metabolism of nitro- and nitrosoaromatics as well as arylamines.

The DNA adducts formed from 1,6- and 1,8-DNP have been well characterized as the corresponding 1-N-(2'-deoxyguanosin-8-yl)-amino-8-nitropyrene and 1-N-(2'-deoxyguanosin-8-yl)-amino-6-nitropyrene adducts, respectively (see Chapter 1). More recently, the mutational spectrum of 1-nitroso-8-nitropyrene, the activated intermediate of 1,8-DNP, was investigated. It was found that the vast majority of the mutations

induced by this toxicant were frameshift mutations (Lambert *et al.*, 1989). None of these characteristics have been established for 1,3-DNP, however (Beland and Kadlubar, 1990). It is thus reasonable to speculate, that the dichotomies observed in the bioactivation and mutagenicity of DNP may not only be due to their differing pathways of metabolic activation, but also due to their mutational specificities. The mutational spectrum of 4-NQO has recently been investigated (Galiegue-Zouitina *et al.*, 1989). Like DNP, 4-NQO is activated by nitro- and nitroso-reductases and preferentially forms N2- and C8-adducts with deoxyguanine. It was established that the induced mutagenicity by the N2-, but not the C8-guanine adduct was dependent on SOS-functions in the host organism *E. coli* and was furthermore dependent on the *umuC* gene product. That chemical mutagenesis may proceed via *umu*-dependent or *umu*-independent mechanisms has also been recognized for 1-NP (Martin and Jennings, 1989) and illustrated in the case of sodium azide (Oda *et al.*, 1987, see Chapter 2). The author therefore suggests that the mutational spectrum of 1,3-DNP may differ from that of 1,6- and 1,8-DNP, as may its metabolic pathways. The *umu*-test relies on the induction of the *umuDC* gene, which is dependent on replication blocks formed in repetitive sequences (Koehl *et al.*, 1989). The response of this assay towards 1,8-DNP, for example, is therefore likely to rely on the preference of 1,8-DNP to form adducts which cause frameshift mutations within such sequences (Lambert *et al.*, 1989). This may not be the case with 1,3-DNP as the additional nitrogroup in the peri-position may effect a different adduct preference. Differences in the effects of rat liver subcellular fractions on the mutagenicity of DNP in the Ames test, particularly those of cytosol, from ethanol- vs. pair-fed animals were apparent in TA100. This was less or not at all the case in TA98 (Traynor, 1988). Note that the 1,3-DNP isomer was the one which displayed the most pronounced effects of ethanol-mediated cytosolic activation in

TA100. Thus, as a consequence of isomer particularities, mutagenic expression may be detectable only using certain mutation-specific Ames-tester strains, but not necessarily employing the *umu*-test.

Chapter 7

QSAR of dinitropyrenes and their proposed metabolites: A theoretical approach.

Experimental investigation of highly reactive mutagens such as activated dinitropyrenes (DNP) is complicated by the unstable nature of such species. Their extreme hydrophobicity further complicates investigations on their bioactivation in a biomimetic environment. The utilization of QSAR (quantitative structure activity relationships) may circumvent some of these problems, hence such a theoretical approach appears to be a highly promising alternative. Nevertheless this approach requires considerable approximation. Even with modern advanced methods of semiempirical calculations the characteristics of the molecule under study are approximated *in vacuo*, which does not allow for considerations pertinent to the action of enzymes on those compounds in a biomimetic environment. The challenge of this approach is to determine whether or not the mutagenic activity of DNP is explainable purely on chemical grounds.

7.1. Introduction

Klopman *et al.* (1984) correlated the energy of the lowest unoccupied molecular orbital (LUMO) with their mutagenicity of a series of nitroarenes that included 1-NP and

DNP. This concept was further evaluated and refined by employing a larger number of nitroarenes (Maynard *et al.*, 1986). While reasonable correlations have been achieved between LUMO energy and mutagenic potential of nitroarenes in the Ames *Salmonella* mutagenicity test, these QSAR seem to hold true only for certain sets of nitroarenes with similar PAH parent structure. The basis of such a link between LUMO energy and mutagenicity is the assumption that the mutagenic potential of any particular nitroarene is directly related to its ease of nitroreduction which, in turn, is thought to be reflected in its LUMO energy. This assumption also implies that the initial nitroreduction is the rate limiting step in the bioactivation of these mutagens and that subsequent metabolism plays a minor role in the formation of the final DNA-binding electrophile. That this assumption is not necessarily true has been discussed by Pryor and coworkers (Shane *et al.*, 1991) who studied the mutagenicity of a series of mono- and dinitrofluoranthrenes. These authors noted that certain nitrated fluoranthrene isomers had virtually identical reduction potentials, yet their mutagenic potency differed by more than two orders of magnitude. It was therefore concluded that "when nitro-PAH of varying steric requirements are compared, the reduction potential may not predict mutagenic potency as well as had been previously suggested". The rate of enzymatic nitroreduction will be affected by parameters other than the reduction potential; steric hindrance, the orientation of the nitrogroup relative to the ring plane, and, hydrophobicity and the dipole moment of the nitroarene are all of importance when considering such enzymatic redox processes.

Debnath *et al.* (1991) reported the structure activity relationships of over 200 mutagenic aromatic and heteroaromatic nitrocompounds and the apparent correlation between mutagenic potential with molecular orbital energies and hydrophobicities. Based on this extensive dataset, these researchers could empirically show that inclusion

of a hydrophobicity factor P into the equation substantially improved the predictive accuracy of the derived QSAR. It was further noted that the mutagenicity was strongly dependent on the number of fused aromatic ring systems in the molecule under consideration; a biphasic curve was obtained which implied that nitroarenes with two or less rings substantially differ from those with a higher number of rings. Those compounds with three or more rings displayed a mutagenicity higher than predicted from their LUMO energy and hydrophobicity alone, which was possibly due to steric reasons (Debnath *et al.*, 1991). Despite of the resulting remarkable correlation, the mutagenicity of certain compounds, including DNP, were severely under- or overestimated. Thus, despite the fact that the mutagenicity of a particular nitroarene may be roughly predicted by its molecular parameters i.e., its LUMO energy and hydrophobicity, specific cases often display an "unpredictive" behavior. The studies by Debnath *et al.* (1991) predicted the mutagenic potential of 1-NP rather accurately. The DNP however, were predicted to be more than 50 times less mutagenic than they actually are and no differentiation in the predicted mutagenic potential between the DNP isomers was apparent.

7.2. Objective

The importance of the nature of the initial nitroreduction step i.e., one- or two-electron transfer and the metabolic consequences associated with these events was emphasized in section 1.4.1¹ The current section describes results obtained from semiempirical AM1² calculations which were designed to investigate the nature of

¹ See also Chapter 4 on the role of NQOR in the reductive metabolism of DNP.

² AM1 stands for Austin method 1 and is an improved version of the highly utilized previous MNDO (Modified Neglect of Differential Overlap) method.

energetically favorable intermediates of all three DNP isomers and 1-NP, in order to unravel possible dichotomies in the theoretically predicted pathways of activation with those observed in their biological activities (Eddy *et al.*, 1986, Howard *et al.*, 1987a). The specific questions to be asked are therefore: *Do the structural and electronic parameters of the three DNP isomers as predicted from semiempirical AM1 computations differ from each other ? What are the parameters of the respective one- and two-electron reduced derivatives of DNP, and finally, do the results obtained from this theoretical approach explain at least some of the differences seen in the metabolic fate and mutagenic characteristics of the respective DNP isomers ?*

7.3. Rationale of design

Mammalian cell fractions contain nitroreductases specific for either one- or two-electron nitroreduction. In order to theoretically predict whether certain DNP isomers are preferentially reduced by either uni- or divalent reduction, heats of formation and molecular orbital (MO) energies were calculated for the series of nitropyrene derivatives (namely 1-NP, 1,3-DNP, 1,6-DNP and 1,8-DNP) as well as their respective one- (1-NP, 1,3-, 1,6- and 1,8-DNP nitro anion radical (1-NAR, 1,3-NAR, 1,6-NAR, and 1,8-NAR, respectively)) and two electron (1-nitrosopyrene (1-NSP) and 1-nitroso-3-, 6-, and 8-nitropyrene (1,3-, 1,6-, and 1,8-NSNP)) reduced products. To the best of our knowledge, this is the first investigation employing quantum mechanical models which considers not only the parent DNP but also their partially reduced derivatives. It is noted that the dinitropyrenes are symmetric about their symmetric planes and reduction of either nitrogroup will lead to identical metabolites.

7.4. Methods

The SCF³ MO calculations were carried out using a micro VAX 3500 employing the AM1 procedure described by Dewar *et al.* (1985) within the MOPAC⁴ program. The MOPAC option, version 5.00 Quantum Chemistry Program Exchange No. 405, Department of Chemistry, Indiana University, Bloomington, IN was used on a SYBYL Molecular Modeling System, Tripos Associates, Inc., St. Louis, MO, Release 5.4. All calculations employed increased precision of convergence and full geometry optimization of all bonds and angles unless otherwise indicated.

7.5. Results

The parameters calculated for the series of nitropyrene derivatives and their partially reduced metabolites employing the AM1 method are shown in table 7.1.

7.5.1 Parent compounds 1-NP and DNP:

The results obtained for the DNP reveal similar MO energies, the difference between either pair of compounds is maximally 0.08 eV (~1.8 kcal/mole) for the LUMO energies. Only the mononitrated pyrene displays remarkably different molecular parameters. This is not unexpected however as the inductive effect of the additional nitrogroup in the DNP will significantly affect the molecular orbital overlap between nitro group and the aromatic ring system. In all four cases studied, the PAH ring system was predicted by the AM1 method to be virtually planar. The predicted

³ SCF stands for self-consistent field.

⁴ MOPAC stands for molecular orbital programs in analytical chemistry.

nitrogroup torsion angles for 1-NP and 1,6- and 1,8-DNP were found to be 27°-30° with respect to the ring plane. In contrast, both nitrogroups in 1,3-DNP were predicted to be close to in plane (3° and 10°) with respect to the aromatic ring system.

We have attempted to evaluate the accuracy of AM1 to predict the nitro torsion angles by comparing the AM1-predicted geometry with that of nitroarenes, for which the crystal structure has been determined. Unfortunately, there are very few examples of those and the only dinitro compound with comparable ring size to that of DNP whose crystal structure has been determined is the vicinal dinitroarene 1,2-dinitrofluoranthrene (Squadrito *et al.*, 1990). AM1 predicted the nitro torsion angles as 53° (for the nitrogroup situated on C1) and 38° (for the nitrogroup situated on C2). The respective values obtained from X-ray crystallography are 73° and 11°, respectively. Thus despite considerable deviations, AM1 seems to correctly predict the torsion angles to be out-of plane and to differ from each other, a probable consequence of the steric clash of the vicinal dinitrocompound (Squadrito *et al.*, 1990). In turn, nitrobenzene is predicted (correctly) to be planar (Dewar *et al.*, 1985). Whether or not DNP do indeed have nitro groups which are not planar with the PAH ring system awaits experimental verification.

Table 7.1. AM1 parameters of mononitro- and dinitropyrenes and their partially reduced metabolites

compound:	H_f (kcal/mole)	dipole moment (Debye)	E_{LUMO} (eV)	E_{HOMO} (eV)	E_{HOMO-LUMO} (eV)
1-nitropyrene	73.60	6.08	-1.70	-8.72	6.02
nitro anion radical	28.01		0.03	-4.83	4.86
1-nitrosopyrene	76.80	3.40	-1.50	-8.44	6.94
1,3-dinitropyrene	84.49	8.58	-2.41	-9.24	6.83
nitro anion radical	21.62	-	-0.75	-5.47	4.75
1-nitroso-3-nitropyrene	84.76	7.03	-2.14	-8.98	6.84

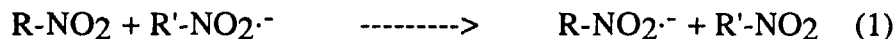
Table 7.1 continued.

compound:	H_f (kcal/mole)	dipole moment (Debye)	E_{LUMO} (eV)	E_{HOMO} (eV)	E_{HOMO-LUMO} (eV)
1,6-dinitropyrene	81.81	0.01	-2.35	-9.30	6.95
nitro anion radical	20.87	-	-0.73	-5.50	4.77
1-nitroso-6-nitropyrene	84.20	2.83	-2.15	-9.01	6.86
1,8-dinitropyrene	82.13	8.10	-2.33	-9.29	6.96
nitro anion radical	21.59	-	-0.71	-5.49	4.78
1-nitroso-8-nitropyrene	85.42	6.75	-2.15	-9.01	6.86

7.5.2 The partially reduced derivatives , nitro anion radicals and nitroso(nitro)pyrenes:

The required computation time for open shell calculations by far exceeds that required for closed shell cases. Initial studies had indicated that no convergence could be achieved employing full geometry optimization of the nitroanion radical species. The geometry of these therefore had to be restricted. To validate this approach, the molecular parameters for 1-NAR were studied as a function of the C₁₁-C₁-N-O torsion angle. In these computations, the torsion angle was fixed in 15° increments from positions of 0° to 90° relative to the aromatic ring plane.

The results obtained from these calculations are shown in Figure 7.1. where it is noted, that the torsion angle predicted for the parent compound coincides, within the accuracy of the experimental approach, with the most stable configuration (minimum in H_f). This observation was taken as sufficient evidence that the nitroanion radical geometries could be approximated from the corresponding geometry of the parent compound. Stabilities and molecular orbital energies for these radical species were therefore calculated employing the AM1 optimized geometries obtained for the parent nitro compounds. The enthalpies for the formation of the radicals (Table 7.3) were calculated according to the isodesmic reaction:



where R-NO₂ and R-NO₂·⁻ are the nitrated pyrene and its corresponding anion radical and R'-NO₂ + R'-NO₂·⁻ are nitromethane and nitromethane anion radical.

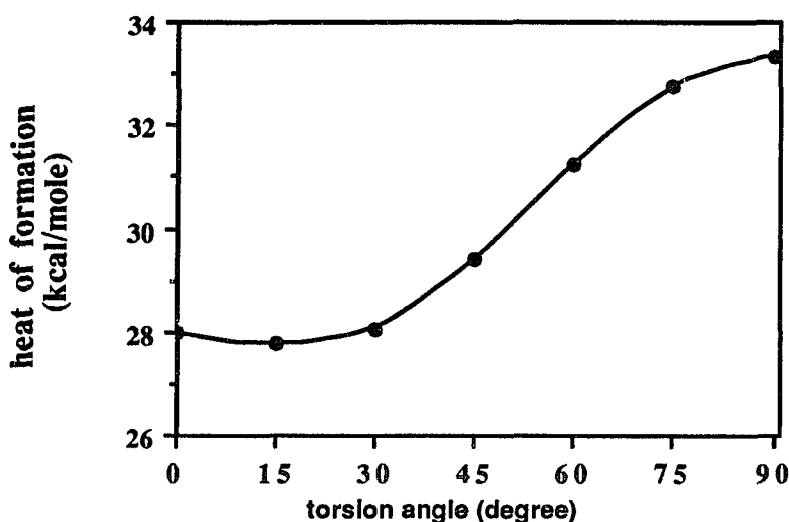


Figure 7.1. AM1-predicted rotational energy barrier for the 1-nitropyrene anion radical. The nitro group torsion angle was fixed in 15° increments from 0°-90° and heats of formation calculated using AM1.

Under thermodynamic control, 1-NAR is predicted to form far more slowly than any of the dinitropyrene anion radicals (Table 7.2). Remarkable however is the lower enthalpy of the radical anion of 1,3-DNP as compared to 1,6- and 1,8-DNP nitro anion radical. Differences of H_f for 1,3-NAR as compared to 1,6- and 1,8-NAR do not exceed 2.3 kcal, however. Predicted E_{LUMO} values for these species are identical within the limits of accuracy of these calculations for the series of dinitropyrene isomers.

The LUMO energies for the three nitrosonitropyrenes were found to be identical to each other. As observed for the parent compound, the LUMO for 1-nitrosopyrene is substantially higher in energy (by approximately 0.65 eV). Thus as observed with the nitro anion radical species, no differentiation between the DNP isomers based on LUMO energies is seen.

Table 7.2 AM1-predicted ΔH_f for nitrated pyrene radicals.

compound	ΔH_f (kcal/mole)
1NAR	-81.00 ^a
1,3-NAR	-98.28
1,6-NAR	-96.35
1,8-NAR	-95.95

^a ΔH_f was calculated according to equation (1) using the AM1 predicted values for nitromethane and nitromethane radical of - 9.33 and 25.48 kcal/mole as given by Korzekwa *et al.* (1990).

The stability of the nitroso(nitro)pyrenes was estimated from ΔH_f which was calculated according to the isodesmic reaction:



where $R-NO_2$ is 1-NP or DNP and $R-NO$ is the respective nitrosopyrene or nitrosonitropyrene.

Table 7.3 AM1-predicted ΔH_f for nitroso(nitro)pyrenes

compound	ΔH_f (kcal/mole)
1NSP	-50.80 ^a
1,3-NSNP	-53.73
1,6-NSNP	-51.61
1,8-NSNP	-50.71

^a ΔH_f were calculated according to the isodesmic reaction (2). The AM1 parameters for water and hydrogen of - 59.2 and - 5.2 kcal/mole, respectively were taken from Dewar *et al.* (1985).

7.6. Discussion:

We have determined the LUMO energies, geometries and stabilities of 1-NP and DNP using the semiempirical MO method AM1. Calculations were also performed to determine the molecular parameters of the respective one- and two-electron reduced species. These partially reduced metabolites are thought to be critical intermediates in the bioactivation and mutagenic expression of nitrated pyrenes. We have taken this approach in order to determine, whether or not semiempirical MO methods such as AM1 would be able to indicate differences in the fate of bioactivation of these compounds, which is reflected in their different mutagenicity towards *S. typhimurium* in the Ames test (see Table 1.1). It should be emphasized that predictions on the metabolic fate and mutagenic potency of chemical mutagens for example by QSAR (Klopman and Rosenkranz, 1984; Klopman *et al.*, 1990; Rosenkranz and Klopman, 1990 a; b), and probably even more so by semiempirical computational methods, is not a trivial matter. This is underlined by the aforementioned lack of correlation between the reduction potentials and mutagenicities of certain nitrated fluoranthrenes.

Eddy *et al.* (1986) employed Hückel calculations and restricted geometries based on the parameters determined for nitrobenzene to calculate E_{LUMO} for 1-NP and DNP. Quantum mechanical molecular models such as MNDO or AM1 offer the advantage of being applicable to complex structures rather than simple models. Applications of this kind still eliminate *ab initio* procedures because of their inaccuracy and/or the enormous computing times required (Dewar *et al.*, 1985). The author preferred the AM1 method over MNDO based on its superiority to make predictions for nitro group and other substituent conformations in nitroaromatic compounds (Dewar *et al.*, 1985). During the course of this study, Debnath *et al.* (1991) have published the AM1-predicted LUMO

energies of the same set of nitrated pyrenes. The LUMO energies reported by these researchers are in good agreement with the values obtained in this study. In contrast to the results obtained by Rosenkranz and coworkers (Eddy *et al.*, 1986; Howard *et al.*, 1987), we have not observed any considerable difference between the LUMO energies of the DNP isomers. Thus, according to our results and those of Debnath *et al.*, (1990) this parameter, which may be taken as an index for the electron affinity, and therefore ease of nitroreduction of the particular compounds under consideration, does not reflect the different rates of nitroreduction between the DNP isomers observed in biological systems. Furthermore, this holds true for the one- as well as the two electron-reduced nitrospecies. This observation is of critical importance, as the rate of formation of the penultimate mutagen (tentatively the hydroxylamino compound) will not only depend on the rate of initial nitroreduction, but also on the rate of reduction or (auto)oxidation of the nitro anion radical or nitrosonitropyrene, whichever one may or may not be formed. We must therefore conclude that the electron affinity as predicted by AM1 does not seem to reflect the metabolic characteristics observed for these DNP isomers *in vitro*. Our results have indicated that while the predicted nitro torsion angles were identical for 1-NP, 1,6- and 1,8-DNP, those of 1,3-DNP were almost in plane with the aromatic ring system. While it is tempting to speculate that this feature may bear some importance in the bioactivation of 1,3-DNP such as the slower rates of nitroreduction, as compared to 1,6- and 1,8-DNP, or its dependence on the classical nitroreductase, amongst other differential effects observed (see Chapter 6), this may be misleading. Our results on the rotational barrier of the 1-nitropyrene radical, which are in good agreement with the calculated rotational energy barriers for other nitrocompounds such as nitrobenzene (Dewar *et al.*, 1985), indicate that a very shallow energy minimum is observed with AM1 for nitrogroup torsion angles between 0-30°.

In the condensed phase, these nitroarenes may have nitro group torsion angles in plane with the PAH ring system.

The calculated ΔH_f values for the formation of the nitroanion radicals indicate that the 1-nitropyrene radical (1-NAR) is more unstable than those derived from DNP. This observation, taken together with the higher LUMO energy of 1-NP is in accordance with the experimentally observed slower reduction of 1-NP, at least as compared to 1,6- and 1,8-DNP (Djuric *et al.*, 1986). 1,3-NAR was predicted to be slightly more stable than 1,6-NAR and 1,8-NAR, as was 1,3-NSNP, compared to 1-NSP, 1,6- and 1,8-NSNP. While this would indicate that rates of formation of 1,3-NAR and 1,3-NSNP would be faster than those of the other isomers, this would only hold true if the reaction is under thermodynamic control, a feature not commonly shared in enzymatic processes.

The relationship between AM1 LUMO energy and observed mutagenicity developed by Debnath *et al.* (1991) dramatically underestimated the mutagenicity of DNP while being rather convincingly accurate for the mononitropyrenes (1-NP and 4-nitropyrene), an observation which parallels that by Eddy *et al.* (1986). Howard *et al.* (1987), based on the earlier studies by Klopman *et al.* (1984) and Eddy *et al.*, (1986), have concluded, that the dichotomies observed in the nitroreduction and mutagenicity of 1-NP and 1,3-DNP, as compared to 1,6-DNP and 1,8-DNP may be due to the preferential one-electron reduction of the former two isomers, compared to the latter. This was based on their respective reduction potentials, their LUMO energies, as well as on the lack of mutagenicity of 1-NP and 1,3-DNP in TA98NR, the nitroreductase-deficient *Salmonella* strain. Our results do not exclude such a relationship, however AM1-predicted LUMO energies do not parallel the different LUMO energies nor, consequently, the different reduction potentials observed by these researchers. Therefore it appears likely, that the metabolic diversities observed in the bioactivation of DNP are not only a direct reflection

of the redox properties of these compounds but also a consequence of the specific characteristics of the enzyme systems involved in the nitroreduction of these isomers.

Summary and Conclusions

The cytosolic metabolism of the three potentially mutagenic dinitropyrene isomers 1,3-, 1,6- and 1,8-DNP has been investigated. Rat liver cytosol was found to catalyze the NADH- and NADPH-dependent reduction of all three DNP. The order of reduction was 1,6-DNP > 1,8-DNP > 1,3-DNP with both cofactors. This relative order, reported previously (Djuric *et al.*, 1986; Heflich *et al.*, 1986), reflects the order of mutagenic potency of DNP in mammalian, but not in bacterial systems (see IARC, 1990 for references). Therefore, nitroreduction may be the rate limiting step in the mutagenic expression of DNP in mammalian, but not in bacterial cells. The semiempirical molecular orbital method AM1 was employed to determine whether specific molecular orbital parameters of DNP predict this order of nitroreduction. Previous studies which employed the Hückel MO approach for determining the same parameters indicated that 1,3-DNP did not have equivalent molecular orbital energies as that of 1,6- and 1,8-DNP (Howard *et al.*, 1987a). This led the authors to conclude that 1,3-DNP is reduced by a one electron mechanism. The present studies indicate that 1,3-DNP is equivalent to 1,6- and 1,8-DNP with respect to orbital energies and therefore can be predicted to undergo reduction by two-electron transferring nitroreductases, for example by NAD(P)H-quinone oxidoreductase (NQOR).

The polychlorinated biphenyl mixture Aroclor-1254 is commonly employed to induce hepatic enzymes which are involved in the activation of a variety of promutagens and procarcinogens. The hepatic subcellular fractions from Aroclor-1254-pretreated rats are thus more active in metabolizing such compounds to the final mutagenic species. We have observed that pretreatment of experimental animals with Aroclor-1254 results

in the induction of cytosolic nitroreductase activity. The sensitivity of the cytosolic nitroreductase activity to dicoumarol suggested that NQOR was involved in the NAD(P)H-dependent reduction of DNP. It was subsequently shown that nitroreductase activity co-purified with NADH-menadione oxidoreductase activity upon affinity purification from rat liver cytosol. NQOR catalyzed the NADPH- and NADH-dependent reduction of all three DNP isomers. The purified enzyme mediated a marked enhancement of the mutagenicity of each of the DNP in the Ames *Salmonella* mutagenicity test. NQOR restored the mutagenic activity of 1,3-DNP in TA98NR, a strain deficient in nitroreductase activity, an observation that is consistent with the results of the AM1 calculations discussed above.

NQOR activity towards menadione and dichlorophenol-indophenol was induced at least 15-fold in cytosol from Aroclor-1254-pretreated rats. This induced activity paralleled the induction of cytosolic DNP nitroreductase activity. Cytosolic dicoumarol-sensitive quinone reductase activity was also induced 3- to 5-fold by chronic ethanol ingestion, yet only slight induction of nitroreductase activity towards DNP was observed. This may be due to the relative velocities of quinone vs. DNP reduction, i.e. DNP reduction is at least two orders of magnitude smaller than menadione reduction in cytosol as well as with purified NQOR. Alternatively, competing nitroreductases or the substrate specificities of NQOR isoenzymes which may be present in rat liver fractions may explain the lower relative induction of DNP vs. quinone reductase activity.

Aroclor-1254 also induces the cytochrome P450 isoenzyme families CYP1A and CYP2B while ethanol ingestion results in the induction of CYP2E. Induction of CYP1A, for example by 3-methylcholanthrene, is mediated by the TCDD-receptor and is accompanied by the induction of NQOR and glutathione-S-transferase (GST) activity (see Nebert *et al.*, 1990). The observed induction of GST and NQOR activity, but not

of CYP1A by chronic ethanol ingestion suggests a different molecular mechanism of gene regulation of this gene battery. We suggest that oxidative stress caused by the cellular metabolism of ethanol may mediate the induction of GST and NQOR as an adaptive response to the increased demand for antioxidant defenses. This would imply that at least two different regulatory regions exist which control the expression of the genes coding for NQOR and GST, which in mice correspond to *Nmo-1* and *Gt-1*, respectively (Nebert *et al.*, 1990). A model accounting for such a regulatory mechanism as a response to oxidative stress has recently been proposed by Nebert *et al.* (1990).

The participation of NQOR in the metabolism and mutagenic expression of DNP may depend on the degree to which the enzyme is induced. The fact that NQOR is present in a variety of organs including heart, lung, stomach and colon (Ernster, 1969; Brunmark and Cadenas, 1989; Siegel *et al.*, 1990 a,b) suggests that this enzyme participates in the metabolism of DNP in organs other than the liver. Furthermore, the activity levels of NQOR differ markedly in different cell lines (Howard *et al.*, 1987a; Siegel *et al.*, 1990 a,b) which suggests that certain cell lines are more susceptible to the genotoxic effects of DNP than others.

Liver cytosol contains nitroreductases other than NQOR. This is indicated by the sensitivity of DNP nitroreduction to allopurinol, an inhibitor of xanthine oxidase, and may also be reflected in the aforementioned lower relative increases of DNP nitroreduction as compared to quinone reduction after pretreatment of the experimental animals with ethanol or Aroclor-1254. One of the metabolic consequences of ethanol ingestion is a decrease in the NAD^+/NADH -ratio from approximately 700 to 200 (Kalant and Khanna, 1980). This may have important implications in the toxic and mutagenic expression of DNP and other compounds which require reduction for activation. NADH is the only pyridine nucleotide donor for xanthine

dehydrogenase/xanthine oxidase (XO/XD). Thus, despite the fact that XO is only slightly induced upon ethanol ingestion (Abbondanza *et al.*, 1989; this work), the increased availability of reduced cofactor may potentiate the rates of nitroreduction of DNP by both NQOR and XO/XD. XO has been shown to catalyze the reduction of 1-nitropyrene to 1-hydroxylaminopyrene (Howard and Beland, 1982) as well as the reduction of 1,6-DNP (Djuric and McGunagle, 1988) under anaerobic conditions in the presence of hypoxanthine. Oxidative metabolism of ethanol leads to the formation of NADH as well as to the formation of acetaldehyde. The cellular concentrations of acetaldehyde formed from ethanol may be too low for this compound to contribute largely as an oxidative substrate for XO, which has a K_m with respect to acetaldehyde of approximately 130 mM (Morpeh, 1983). Under conditions of strong ethanol intoxication however, it may serve as a substrate for aldehyde oxidase, which has a much lower K_m (1 mM) for this compound (Rajagopalan and Handler, 1964). Aldehyde oxidase has been suggested to be involved in the reduction of DNP (Djuric *et al.*, 1986), thus ethanol oxidation may result in increased rates of nitroreduction of DNP by providing oxidative substrate for aldehyde oxidase.

NQOR catalyzes the reduction of a variety of C-nitroso compounds (Horie *et al.*, 1982, this work). Therefore, ethanol ingestion is likely to increase the rates of both nitro- and nitroso reduction by the process of enzyme induction and increased cofactor availability. The resulting hydroxylamino compounds may then be acetylated by cytosolic acetylCoA-dependent N-acetyltransferases (NATase). While we observed that ethanol ingestion does not alter NATase activity, increases in cellular acetylCoA concentrations have been reported following ethanol ingestion (Bode *et al.*, 1970; Kondrup and Grunnet, 1973). This would only hold true however, if the acetylCoA concentrations are well below the K_m of the acetyltransferase(s) for this cofactor.

We and others (Nakamura *et al.*, 1987; Shimada and Guengrich, 1990) have observed that DNP potently induced SOS-dependent induction of the *umu*-gene in *Salmonella typhimurium* TA1535/pSK1002. The order of potency was 1,8- > 1,6- > 1,3-DNP which follows the order of mutagenic potency in the Ames *Salmonella* mutagenicity assay. Addition of rat liver cytosolic fractions in the presence of an NADPH-generating system strongly deactivated 1,6- and 1,8-DNP, but activated 1,3-DNP. The opposite was true when microsomes were used as the catalytically active fraction. Microsomal deactivation followed the order 1,3- > 1,6- > 1,8-DNP, thus underlining the isomer specificity of the microsomal and cytosolic enzymes involved in the metabolism of these compounds. In all cases the 1,3-DNP isomer, which is the only one containing both nitrogroups in the same aromatic ring, displayed very different characteristics than did the other two DNP isomers. We suggest that this disparity in the metabolism of DNP isomers might be due not only to differing rates of nitroreduction, but also to the relative ratios of nitro- and nitroso-reduction versus acetylation. Furthermore, it cannot be excluded that 1,3-DNP displays a different DNA adduct preference than 1,6- and 1,8-DNP. Both 1,6- and 1,8-DNP have been shown to primarily form an adduct at the C-8-position of guanine, whereas the adduct of 1,3-DNP has not been characterized (Beland and Kadlubar, 1989). Our result in the *umu*-test underline the importance of varying protein- as well as toxicant concentrations when assessing the effects of mammalian fractions on the bacterial mutagenicity of toxicants which are metabolically activated by both, bacterial and mammalian enzymes. In conclusion, rat liver cytosol and microsomes contain enzymes which can activate or deactivate DNP. The mutagenic expression of DNP will vary as a function of animal pretreatment and isomer preference of the enzymatic activities which are induced by that pretreatment.

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Appendix

Appendix 1.

Dicoumarol is an inhibitor of xanthine oxidase

Abstract

Dicoumarol was found to be a mixed type inhibitor of the xanthine oxidase-catalyzed oxidation of xanthine to urate. This coumarin analogue inhibited hypoxanthine- and xanthine oxidation to similar extents and a K_i apparent of 5 μM was determined from secondary plots with xanthine as the substrate. The K_i value was similar to that of the K_m of xanthine oxidase for its substrate xanthine. The results indicate, that caution should be exercised when dicoumarol is used as an inhibitor of NAD(P)H-quinone oxidoreductase (NQOR) (DT-diaphorase, [E.C. 1.6.99.2]).

Introduction

Dicoumarol (3,3'-methylene-bis(hydroxycoumarin), DC)) is a well-recognized anticoagulant owing to its ability to interfere with vitamin K-metabolism (Ernster, 1969; Suttie, 1985). It is also commonly used in standard assays of NQOR in cytosolic and microsomal fractions as a selective inhibitor to demonstrate the presence or absence of this enzyme in biological tissues (Märki and Martius, 1960; Ernster, 1969; Nims *et al.*,

1984; Lind *et al.*, 1978). It is commonly used in studies designed to show induction of NQOR (Benson *et al.*, 1980; Lind *et al.*, 1982; Hajos and Winston, 1991) or to implicate a role for this enzyme in reductive metabolism (Lind *et al.*, 1978; Morrison *et al.*, 1986; Powis *et al.*, 1987; DeFlora *et al.*, 1988; Parola *et al.*, 1990; Belisario *et al.*, 1990; Preusch and Smalley, 1990). In studies designed to implicate a role for NQOR in the reduction of nitroaromatic compounds we observed that DC inhibited cytosolic xanthine oxidase (Hajos and Winston, 1991), another flavin-containing enzyme which catalyzes the reduction of a wide variety of compounds. A role for xanthine oxidase [E.C. 1.2.3.2.] in oxyradical-mediated tissue damage associated with reperfusion injury is increasingly reported (McCord and Roy, 1982). Superoxide anion radical and hydrogen peroxide are both by-products of numerous reactions catalyzed by xanthine oxidase (Fridovich, 1970; Olson *et al.*, 1974; Kappus and Sies, 1981; Winston *et al.*, 1984; Nagano and Fridovich, 1985; Gutteridge *et al.*, 1990; Bounds and Winston, 1991). In contrast to xanthine oxidase, which mediates oxidative tissue damage, NQOR is thought to protect tissues from such damage by virtue of its ability to divalently reduce a wide variety of inorganic and organic substrates e.g., metals, quinones and nitroaromatic compounds (Smith *et al.*, 1985; Lind *et al.*, 1982), thereby preventing the formation of reactive oxygen species that are formed upon autoxidation of these substrates after univalent reduction.

Materials and Methods

Dicoumarol, diethylene triaminopentaacetic acid (DTPA), hypoxanthine, tetrasodium pyrophosphate, xanthine and xanthine oxidase (grade 1 from buttermilk) were from Sigma Chemical Company, St. Louis, MO. Dicoumarol stock solutions

were prepared freshly in distilled water and just enough 10 N NaOH to dissolve the compound. Xanthine oxidase (0.59 units/mg as an ammonium sulfate suspension) was desalted by gel chromatography on a prepacked Sephadex G-25 column (Pharmacia, Uppsalla, Sweden) prior to use. Activity of the enzyme was determined with 50 μ M hypoxanthine or 5-50 μ M xanthine in the presence of 0-200 μ M dicoumarol. Substrate, inhibitor and enzyme were added to 50 mM sodium pyrophosphate buffer, pH 7.5 containing 0.1 mM DTPA in a final volume of 1 ml. The change in absorbance at 295 nm due to urate formation was followed using a Perkin Elmer lambda 5 spectrophotometer with water-jacketed quartz cuvettes at 30°C. One activity unit is defined as the amount of enzyme which causes a change of one optical density unit per minute at 295 nm in the presence of 50 μ M xanthine (Rajagopalan, 1985).

Results and Discussion

Initial experiments were designed to evaluate the effect of increasing concentrations of DC on the xanthine oxidase-catalyzed oxidation of hypoxanthine or xanthine to urate at saturating levels of substrate (50 μ M). As shown in Figure 1, DC inhibited the conversion of both substrates to approximately equal extents. No change in absorbance at 295 nm was observed over time in the presence of 100 μ M DC but the absence of hypoxanthine or xanthine. Concentrations of DC above 100 μ M however, caused a high background absorbance at 295 nm due to the absorbance of the inhibitor at this wavelength. This interfered with the accurate determination of the rate of urate formation by the spectrophotometric method employed. For the kinetic analysis therefore, concentrations below this DC concentration were employed.

DC (50 μM) was equally inhibitory over the range of 40-400 mU of XO, conditions under which the reaction was linear with respect to protein (not shown).

The inhibition kinetics of xanthine oxidase by DC were determined with xanthine as the substrate (5-50 μM) in the absence or presence of varying fixed concentrations of DC. These results are shown in the Lineweaver-Burk plots in Figure 2. All plots converge on a single point behind the y-axis indicating that DC exhibits a mixed mode of inhibition. A replot of the slopes versus the inhibitor concentration (Figure 2, inset) reveals the K_i app. to be $\sim 5 \mu\text{M}$ for dicoumarol.

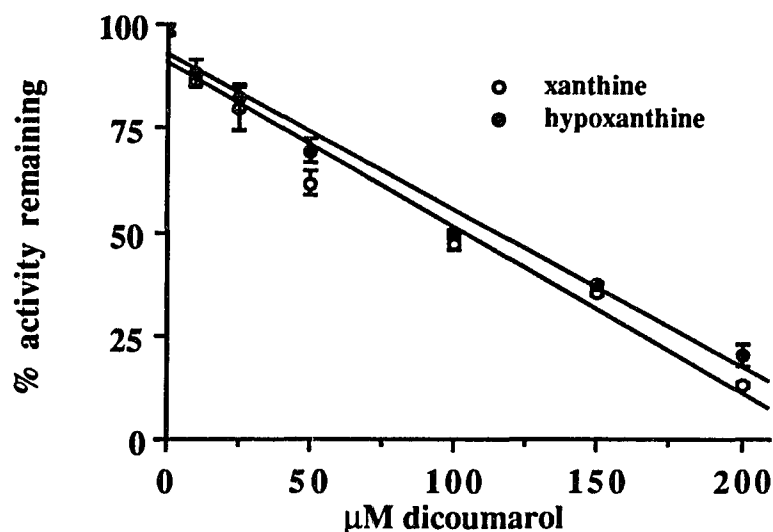


Figure 1 Xanthine oxidase (~ 300 mU) was incubated in the presence of 50 μM hypoxanthine or xanthine and 0-200 μM dicoumarol and the rate of formation of urate was determined spectrophotometrically. Results are presented as percent activity compared to uninhibited control and are the mean of triplicate determinations at each DC concentration.

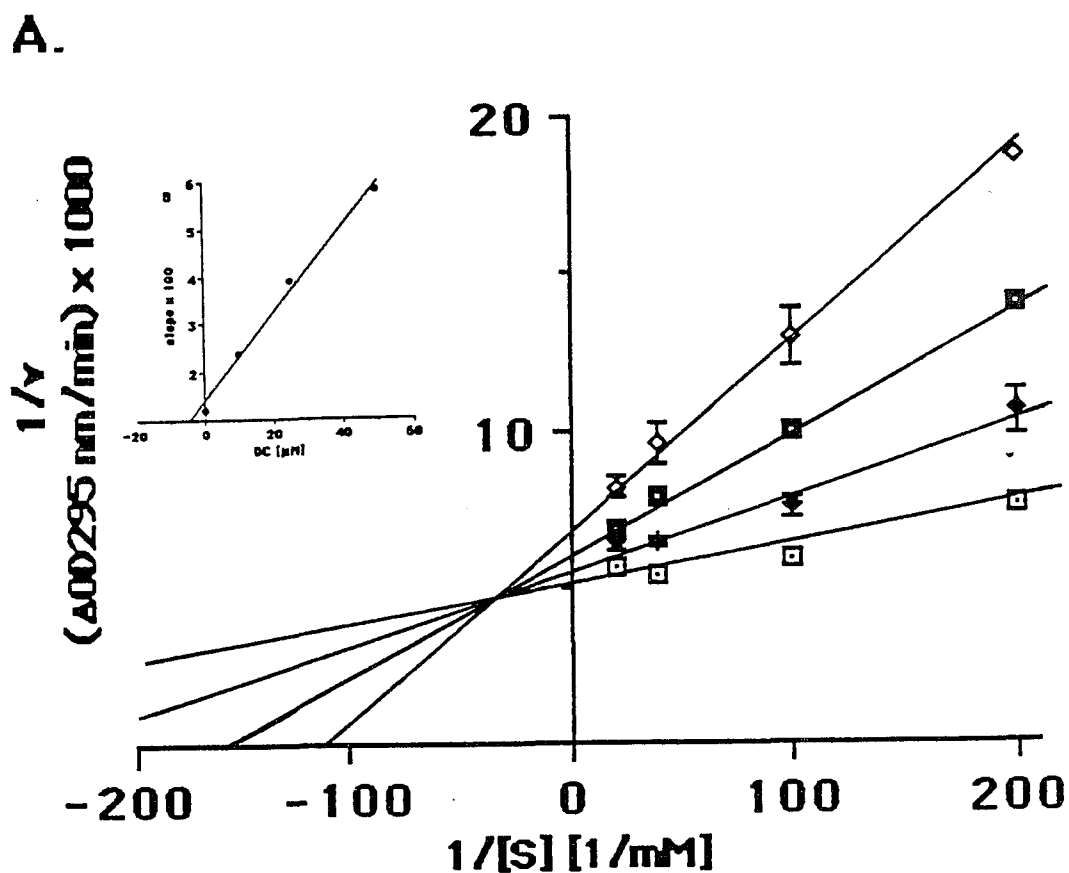


Figure 2 Double-reciprocal plots of the xanthine oxidase-catalyzed formation of urate: effect of varying the concentration of dicoumarol (A). Purified xanthine oxidase was incubated in the presence of 5-50 μM xanthine and 0, 10, 25 and 50 μM dicoumarol. The rate of formation of urate was determined spectrophotometrically. Each data point represents the mean \pm SD of triplicate determinations. A replot of the slopes vs. the DC concentration is shown in the inset (B).

The presented results show that DC is a mixed-type inhibitor of xanthine oxidase with a K_i similar to the K_m of the enzyme for xanthine (Olson *et al.*, 1974; Nagano and Fridovich, 1985). Results obtained with this inhibitor may be subject to mendacious interpretation as DC may in fact be inhibiting XO. Interpretation of results relying on the specificity of this inhibitor may be further complicated by the fact that both xanthine oxidase and NQOR are greatly variable depending on the source of the enzyme fraction under study and the pretreatment of the experimental animal (Benson *et al.*, 1982; Nims *et al.*, 1984; Abbondanza *et al.*, 1989; Livingstone *et al.*, 1990; Hajos and Winston, 1991). A variety of substrates are common to both of these enzymes including sundry aldehydes (Bounds and Winston, 1991; Gutteridge *et al.*, 1990), aromatic ketones and heteroaromatics (Ernster, 1969; Preusch and Smalley, 1990) and aromatic nitro- (DeFlora *et al.*, 1988; Howard and Beland, 1982; Hajos and Winston, 1991) and nitrosocompounds (Horie *et al.*, 1982). The assignment of an activity to catalysis by NQOR based solely on the sensitivity of the reaction to DC is insufficient and would require more rigorous characterization.

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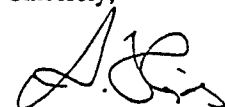
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Vita

Antal K. D. Hajos was born on March 20, 1964 in Innsbruck, Austria. He is the son of Prof. Dr. Anton Hajos and Dr. Elisabeth Hajos. He graduated from high-school (Landgraf-Ludwig Gymnasium, Giessen, FRG) in 1983 and earned his B.S. equivalent (Vordiplom I and II in physiological chemistry) at the Eberhardt-Karls University Tübingen, FRG in 1985. He has performed undergraduate research in the laboratories of Dr. W. Voelter at the Institute for Biochemistry and under supervision of Dr. H. Frank in Dr. H. Remmers laboratories at the Institute for Toxicology, both at the University of Tübingen, FRG. He was awarded a full joint scholarship from the University at Tübingen and Louisiana State University, Baton Rouge, LA to study at LSU in 1986 and is currently a Ph.D. candidate in the Biochemistry Department at this University. He is a member of the Society for Free Radical Research, the Oxygen Society, Phi Lambda Upsilon and the Universitätsbund Tübingen.

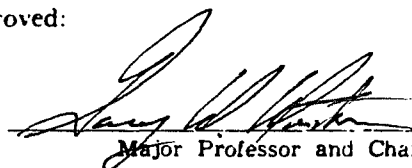
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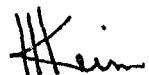
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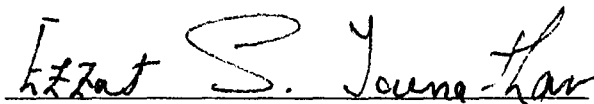
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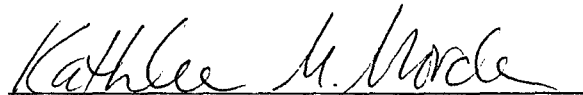
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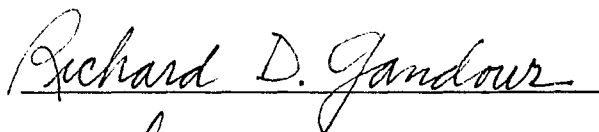

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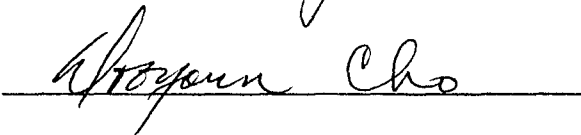

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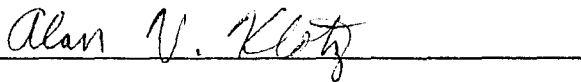
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